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MANUAL OF

VETERINARY BACTERIOLOGY

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VETERINARY BACTERIOLOGY

By

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BALTIMORE
THE WILLIAMS & WILKINS COMPANY
1927

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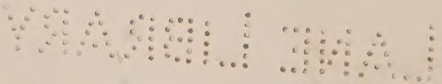
Made in United States of America

Published June, 1927

COMPOSED AND PRINTED AT THE
WAVERLY PRESS
FOR
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, MD., U. S. A.



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PREFACE

Text books of veterinary bacteriology are very limited in number. Of the few that have been published the author knows of but one in the English language which is of fairly recent date. Thus, in offering the present volume, it is hoped that the same will, in a small way at least, serve to supplement the few works already available.

The classification of bacteria and bacteriological nomenclature are, at the present time, subjects of considerable concern. While a number of bacteriologists have adopted the classification and nomenclature advocated by the Society of American Bacteriologists, some workers in this field of science adhere to the old system of grouping bacteria and continue to employ the older terms. Such situation is, obviously, far from satisfactory. The importance of uniformity in classifying bacteria, and particularly in the use of names of the various species, is such that it admits of no argument, yet a casual perusal of current bacteriological literature will reveal a deplorable lack of uniformity in such respect.

For years bacteriologists have recognized the need for a more adequate means of classifying bacteria. This need prompted the Society of American Bacteriologists to appoint a committee to study the situation and develop a more satisfactory classification. The outgrowth of the committee's study and activity has been the development of a rather elaborate system for the identification and classification of bacteria. Such system is incorporated in "Bergey's Manual of Determinative Bacteriology," prepared by the committee consisting of Bergey, Harrison, Breed, Hammer and Huntoon.[1] The first edition of this work was published in 1923. A second edition appeared in 1925.

While the classification and nomenclature advocated by the Society of American Bacteriologists' committee may not be flawproof and will, undoubtedly, from time to time, be improved upon, it is certainly a distinct advance over what we have previously had. Thus, in the preparation of this volume such system has been adopted. Care has been taken, however, to include the older names of bacteria as synonyms and in the index both old and new terms appear.

[1] Bergey's Manual of Determinative Bacteriology, 2nd ed., 1925, published by The Williams & Wilkins Co., Baltimore, Md.

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In treating of the different bacteria the various species are arranged in chapters according to genus. Each chapter heading, besides indicating the genus to which the species treated of belong, also indicates the position of the particular genus in the system as a whole.

This work has been prepared primarily for the use of those concerned with veterinary bacteriology, hence, no consideration has been given to organisms not of interest in veterinary medicine.

In the section on infection and immunity no attempt has been made to present a full discussion of the subject generally. A detailed treatise of all phases of infection and immunity would require an entire volume. This work aims only at giving a few of the principles and theories which have served as a basis for the development of the subject.

While not in conformity with the title of the book, sections have been included on protozoa and hematology. The importance of a working knowledge of the protozoan organisms which may be encountered by the bacteriologist in his investigation of disease and disease processes is obvious, hence, the customary practice of devoting space to some of the more important animal parasites has been followed. As the task of making clinical blood examinations commonly devolves upon the bacteriologist, a short section on hematology has been included.

The first chapter of the section on protozoa, dealing with the subject generally, is from the pen of Prof. Frank G. Haughwout, Protozoölogist of the Bureau of Science, Manila, P. I. The author takes this opportunity to gratefully acknowledge his indebtedness to Professor Haughwout for such chapter.

I am also deeply indebted to Captain Francois H. K. Reynolds, V. C., Officer in Charge, Veterinary Laboratory Section, Army Medical School, Washington, D. C., for a large number of the pen drawings and the colored plates.

Thanks are due to Sir Arnold Theiler, Director of Veterinary Education and Research, Union of South Africa, Pretoria, S. A., for furnishing various preparations of protozoan organisms for use in making photomicrographs.

With a few exceptions the photomicrographs of the various organisms illustrated are by the author. For the others I am indebted to Major George R. Callender, M.C., Curator of the Army Medical Museum, Washington, D. C.

The author is also indebted to his wife for assistance in connection with the preparation of the manuscript and the reading and correcting of proof.

Finally, I would be lax in my duty, did I not acknowledge and express

appreciation of the care and painstaking efforts exercised by the publishers in putting the manuscript in book form.

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RAYMOND A. KELSER.

CONTENTS

PART I

Bacteria, Their Morphology, Physiology and Classification

Chapter I.—Introduction..... 3
Chapter II.—Morphology, Physiology and Classification..... 8

PART II

Bacteriological Methods

Chapter III.—The Microscope..... 25
Chapter IV.—Sterilization, Preparation of Glassware, etc..... 31
Chapter V.—Preparation of Culture Media..... 44
Chapter VI.—Methods Employed for the Artificial Propagation of Bacteria..... 65
Chapter VII.—Microscopic Study of Bacteria; Stains and Staining Methods..... 83

PART III

Infection and Immunity

Chapter VIII.—Microörganisms and Disease; Resistance and Immunity..... 101
Chapter IX.—Theories of Infection and Immunity; Phagocytosis; Opsonins and Opsonic Index; Antibodies..... 105
Chapter X.—Hypersensitiveness (Allergy—Anaphylaxis)..... 119

PART IV

Pathogenic Organisms of the Class Schizomycetes

Chapter XI.—Order—Eubacteriales, Family—Coccaceae, Tribe—Streptococceae. *Organisms of the Genus Streptococcus*..... 127
Chapter XII.—Order—Eubacteriales, Family—Coccaceae, Continued, Tribe—Micrococceae. *Organisms of the Genus Staphylococcus*..... 141
Chapter XIII.—Order—Eubacteriales, Continued, Family—Spirillaceae. *Organisms of the Genus Vibrio*..... 146
Chapter XIV.—Order—Eubacteriales, Continued, Family—Bacteriaceae, Tribe Chromobacterieae. *Organisms of the Genus Pseudomonas*..... 149
Chapter XV.—Order—Eubacteriales, Family—Bacteriaceae, Continued, Tribe—Bacterieae. *Organisms of the Genus Escherichia*..... 152
Chapter XVI.—Order—Eubacteriales, Family—Bacteriaceae, Tribe—Bacterieae, Continued. *Organisms of the Genus Salmonella*..... 157
Chapter XVII.—Order—Eubacteriales, Family—Bacteriaceae, Tribe—Bacterieae, Continued. *Organisms of the Genus Eberthella*..... 171
Chapter XVIII.—Order—Eubacteriales, Family—Bacteriaceae, Tribe—Bacterieae, Continued. *Organisms of the Genus Alcaligines*..... 175

Chapter XIX.—Order—Eubacteriales, Family—Bacteriaceae, Continued, Tribe—Pasteurelleae. *Organisms of the Genus Pasteurella* 186
Chapter XX.—Order—Eubacteriales, Continued, Family—Bacillaceae. *Organisms of the Genus Bacillus* 201
Chapter XXI.—Order—Eubacteriales, Family—Bacillaceae, Continued. *Organisms of the Genus Clostridium* 213
Chapter XXII.—Order—Actinomycetales, Family—Actinomycetaceae. *Organisms of the Genus Actinobacillus* 242
Chapter XXIII.—Order—Actinomycetales, Family—Actinomycetaceae, Continued. *Organisms of the Genus Actinomyces* 244
Chapter XXIV.—Order—Actinomycetales, Family—Actinomycetaceae, Continued. *Organisms of the Genus Erysipelothrix* 258
Chapter XXV.—Order—Actinomycetales, Continued, Family—Mycobacteriaceae. *Organisms of the Genus Mycobacterium* 263
Chapter XXVI.—Order—Actinomycetales, Family—Mycobacteriaceae, Continued. *Organisms of the Genus Corynebacterium* 285
Chapter XXVII.—Order—Actinomycetales, Family—Mycobacteriaceae, Continued. Genus Pfeifferella. *Pfeifferella mallei* 289
Chapter XXVIII.—Order—Spirochaetales, Family—Spirochaetaceae. *Organisms of the Genus Borrelia* 295

PART V

Pathogenic Fungi

Chapter XXIX.—Blastomycetes and Hyphomycetes 301
Chapter XXX.—Pathogenic Blastomycetes 305
Chapter XXXI.—Ascomycetes 309
Chapter XXXII.—Fungi Imperfecti 317

PART VI

The Protozoa

Chapter XXXIII.—The Parasitic Protozoa; Their Mode of Life and Relation to Their Hosts; Some Technical Considerations 323
Chapter XXXIV.—Sarcodina 361
Chapter XXXV.—Mastigophora 362
Chapter XXXVI.—Sporozoa 374
Chapter XXXVII.—Infusoria 389

PART VII

The Filterable Viruses

Chapter XXXVIII.—Disease-producing Viruses Capable of Passing Bacteria-retaining Filters 393

PART VIII

Serology

Chapter XXXIX.—Serological Tests Commonly Employed in Veterinary Medicine . 421

PART IX

Hematology

Chapter XL.—Clinical Blood Examinations; Enumeration of Blood Cells; Estimation of Hemoglobin; Differential Counts; Normal Blood of Domestic Animals.. 453

PART X

Preparation of Veterinary Biological Products

Chapter XLI.—The Production of Bacterins, Vaccines, Aggressins, Sera, Antitoxins, Tuberculin, Mallein, etc..... 463

PART XI

Bacteriological Examination of Milk and Water

Chapter XLII.—Examination of Milk; Certified Milk; Pasteurization; Examination of Water..... 487

PART I

BACTERIA, THEIR MORPHOLOGY, PHYSIOLOGY AND CLASSIFICATION

CHAPTER I

Introduction

No other science has contributed more to the welfare of mankind than bacteriology. The importance of modern medicine, surgery, sanitation, etc., in our present-day order is universal common knowledge. The development of these sciences was made possible largely through the revelations of bacteriology. Thus, bacteriology has supplied the key to many problems of health, agriculture and industry and has served to pave the way for some of the greatest of scientific advances. It is gratifying knowledge that in the general development of this important science veterinary bacteriology has played a large part. As will be noted in the following paragraphs some of the epoch-making findings in bacteriology have resulted from toil in the field of veterinary bacteriology.

The discovery of bacteria was, obviously, dependent upon the development of an instrument capable of magnification sufficient to bring such organisms within the range of visibility. While the evidence is not absolutely definite, a Holland family by the name of Janssen is generally accredited with the invention of the first microscope at about the end of the sixteenth century. Their instrument was very crude and capable of magnifying only a few diameters.

But little progress was made with the perfection of the microscope until 1675 when Antony van Leeuwenhoek, also of Holland, produced the first such instrument of importance. With this microscope, which was capable of magnifying about 150 diameters, Leeuwenhoek detected minute forms of life in saliva, water and decomposing organic matter. This marked the beginning of microbiology. It should be noted, however, that Leeuwenhoek was not the first to observe microscopic organisms. As early as 1659 Athanasius Kircher, with a very crude lens, saw, in various types of putrid material, minute, living bodies not discernible to the naked eye. His observations, however, received but very little attention. Since those early days the microscope has been gradually improved so that at the present time we can satisfactorily magnify objects 1500 or more diameters.

Following the researches of Leeuwenhoek a number of investigators were quick to take up the study of the microscopic forms of life but for a long time nothing of particular importance was recorded.

In 1762 Plenciz, of Vienna, expressed the belief that the minute forms seen in saliva, water, feces, etc., were responsible for certain of the infectious diseases. The views of Plenciz and his followers did not gain ground, due, in a large measure, to the contention which had arisen concerning the origin of these microscopic organisms which were attracting so much attention.

Several prominent investigators, notably Needham and Van Helmont, had propounded the theory of spontaneous generation. On the other hand, another group of equally prominent scientists, headed by Spallanzani, contended against such theory. Both factions conducted various types of experiments with a view to supporting their particular contentions. Needham found that when meat was placed in a flask, sealed, and then put in boiling water, it later developed large numbers of bacteria. Spallanzanii, on the other hand, showed that by more prolonged boiling bouillon in sealed flasks remained sterile. Spallanzani's work, however, was not accepted as definite proof of the fallacy of the spontaneous generation theory as it was held that excessive heating and protection from air resulted in conditions not conducive to the production of bacterial life.

Schultz, another opponent of the spontaneous generation theory, in an effort to meet the criticism directed at Spallanzani's experiments, passed air through sulphuric acid into sterile bouillon and demonstrated that the latter remained sterile. Then, Theodore Schwann, also an opponent of the spontaneous generation theory, permitted air to enter a flask of sterile bouillon by way of a tube heated to a high temperature by direct flame and demonstrated that the bouillon remained sterile. These experiments were likewise criticized by the proponents of the spontaneous generation theory on the ground that the changes brought about by such treatment of the air prevented the development of microörganisms. In refutation of this contention Schroeder and Dusch later demonstrated that plugging a flask of sterile bouillon with a cotton fiber plug, which merely acted as a filter for the air, would result in the bouillon remaining sterile. This also failed to convince and in spite of all experiments the controversy continued until 1860 when Pasteur took it up.

Pasteur carried out a series of experiments in which he showed that where bouillon in a flask was permitted to be in contact with air by means of a "U" tube no contamination occurred, but, that if the fluid was permitted to run down in the bottom of the tube by slanting the flask growth resulted. He also demonstrated that a single fiber of cotton from a quantity which had served as an air filter, when dropped into sterile bouillon, resulted in the growth of a large number of organisms in a very short time. Pasteur also proved that boiling would not destroy all microörganisms, some resisting

the process for a long period of time. It remained for Cohn, however, several years later, to explain this latter observation of Pasteur's by calling attention to bacterial spores and their more resistant qualities. Pasteur's work definitely eliminated the spontaneous generation theory and paved the way for important research which followed.

As far back as 1838 Cagniard-Latour and a little later Theodore Schwann, had expressed the belief that yeast cells found in fermenting alcoholic beverages were the cause of the fermentation process. But little notice had been given this subject because of the attention the spontaneous generation theory was attracting. Pasteur, however, had also devoted study to the fermentation of beer and wine and was able to definitely affirm the views of Cagniard-Latour and Schwann.

With the spontaneous generation theory definitely out of the way and the cause of fermentation determined, interest was again revived in the possible relationship of bacteria to the various infectious diseases. The analogy of certain disease processes to fermentation had long been recognized. Robert Boyle, back in the seventeenth century, had predicted that when the mystery of fermentation was solved much light would be thrown on the cause of infectious diseases. Thus, with fermentation shown to be a bacterial process the possibility of infectious diseases having a like origin became apparent.

In 1855 Pollender, who had been studying anthrax, reported the observation of rod-shaped structures in the blood of animals dead of the disease. Such rods were later observed by Davaine and by Rayer. The discovery was also confirmed by Brauell who further demonstrated the presence of the rods in the blood of living animals in the late stages of anthrax. It remained for Davaine, however, in 1863, to furnish convincing proof of the relationship of the organism to the disease. He demonstrated that anthrax could be regularly produced by the inoculation of susceptible animals with blood containing rods, even when such blood was greatly diluted, and that the disease could not be produced with blood which did not contain such rods. The organism was subsequently isolated in pure culture by Koch (1876) and the disease produced with such cultures, thus establishing beyond all doubt the etiological significance of the organism. Thus, anthrax was the first disease definitely shown to be of bacterial origin.

New achievements and discoveries now came rapidly. Bacterial agents in puerperal sepsis, abscesses and other pyogenic processes were observed by various investigators. Lister (1875) inaugurated antiseptic procedures in the treatment and prevention of wound infections. Such procedures have played a major part in the development of modern surgery. In 1878

Bollinger, in studying material from a tumor-like process on the jaw of a cow, discovered the "ray fungus" (Actinomyces) of actinomycosis. In the same year Perroncito observed the causative agent of fowl cholera. The year 1878 also witnessed Koch's discovery of the organism of mouse septicemia. This same investigator, four years later (1882), presented his epoch-making findings in tuberculosis.

Pasteur, in a communication to the Academy of Medicine in Paris in 1880, presented the results of his immunization experiments with fowl cholera. This constituted the first instance of artificial immunization against an infectious disease in which the nature of the causative factor was known and in which pure cultures entered into the preparation of the immunizing agent. It should be recalled, however, that Jenner, more than eighty years ahead of Pasteur's work with fowl cholera, demonstrated reciprocal immunity between smallpox and cowpox and initiated the method of vaccination which has served to eliminate variola as a serious scourge of mankind. Shortly after his communication on fowl cholera, Pasteur presented his classical work on anthrax immunization and rabies prophylaxis.

In the meantime, through the researches of Weigert, Ehrlich and Koch, the use of aniline dyes was introduced into bacteriology. This made a more accurate study of the structure of bacteria possible and, further, through variations in the staining characteristics of certain organisms, proved a help in the identification of certain forms.

In 1880 Griffith Evans, working with surra in India, discovered in the blood of affected animals, the trypanosome responsible for the disease. This was the first instance in which a trypanosome was shown to be responsible for an infectious disease. As a matter of fact, this was the first account of a protozoan organism producing a specific infectious disease in the higher animals. Pasteur in 1865, however, proved a disease of silkworm to be due to a protozoan organism.

A tremendous step forward was made in 1882 when Koch inaugurated the use of solid culture media. This made the isolation of bacteria in pure culture a simple procedure and, further, afforded a means for the study of colony formation, pigment production, etc., thus greatly facilitating the study and identification of bacteria.

Another advance in 1882 was Laveran's discovery of the plasmodium of malaria in man.

In 1885 Nicolaier observed the tetanus organism in animals inoculated with garden earth and two years later Rosenbach reported the finding of the same organism in a wound in a man who had developed tetanus.

These investigators, however, were unable to cultivate the organism. In 1889 Kitasato, by producing strict anaerobic conditions, was able to grow the tetanus bacillus. This finding, obviously, was another marked advance as it paved the way for isolation and cultivation of the various anaerobes. During the same year Roux and Yersin discovered that the diphtheria organism produced a specific, soluble toxin. Two years later Kitasato found that C. tetani likewise functioned through the production of a powerful, soluble toxin. Then came the announcement of the discovery of diphtheria and tetanus antitoxins by von Behring and his associates Kitasato and Wernicke.

In 1888 Babes, in Roumania, studying the disease now known as "bovine piroplasmosis" or "Texas fever," observed diplococcus-like bodies in the red blood corpuscles of affected animals. He had observed the protozoan organism responsible for the disease but failed to recognize its exact nature. In 1893 Smith and Kilborne of the United States Bureau of Animal Industry, proved the protozoan nature of the Texas fever organism and demonstrated that it was transmitted from animal to animal by ticks. This was the first instance in which an insect was proven to be the intermediate host and transmitting medium of the agent of an infectious disease. Medical entomology dates from this finding.

Bacteriology has thus developed by leaps and bounds. Numerous organisms have been identified as the causative factors of various diseases, bacteriological methods and technic have been perfected, various tests developed, vaccines and sera produced for use in the prevention and treatment of disease, and numerous other advances made which have contributed tremendously to the well-being of man and beast. However, with all of its achievements the science is barely out of its infancy and the field but partially explored.

CHAPTER II

Morphology, Physiology and Classification

Bacteria are microscopic, unicellular plants occurring in the shape of *rods* (bacilli), *cocci* (spheres), and *spirilla*. They are among the very lowest forms of life, in fact being so simple in structure that they have caused considerable concern as to whether they belong to the animal or plant kingdom. While they differ from the higher plants in several ways, such as lacking chlorophyl and possession of motility by some forms, it is at present quite generally accepted that they should be included in the vegetable kingdom. Bacteria are widely distributed in nature. They occur everywhere throughout the world. Fortunately, but a small percentage of them are pathogenic for man and lower animals.

The *rod* (bacillus) forms vary in size from those barely perceptible to types as long as 50 microns. They may be short and broad, short and narrow, long and broad, long and narrow, or intermediate between such forms. The ends may be convex, slightly concave, or square-cut. They may be non-sporulating or they may form central or end spores. Some forms possess a demonstrable capsule, others do not. Some are provided with flagella which give them motility while other, non-motile forms have no flagella. The flagella, and usually the capsules of bacteria, are not seen in the ordinary preparation, special technique being required to bring out such structures. The rods are found singly, in pairs, or in short or long chains.

The *cocci* (spheres) generally appear as round bodies of varying size, but occasionally they occur as slightly oval or even angular bodies. They are non-motile, do not form spores and most of them are non-capsulated. They are found in pairs (diplococci), in short or long chains (streptococci), in grape-like clusters (staphylococci), in groups of four (tetrads), or in cubes (sarcina).

The *spirilla* are short and narrow, short and broad, long and narrow, long and broad, or intermediate between these. They may possess but one curve making them comma-shaped, may have several curves giving them an S-shaped appearance, or they may possess numerous curves giving them a cork-screw appearance. They are motile by means of flagella with the exception of certain spirochaetes which owe their motility to a type of undulating membrane.

Transmutation. While there is no evidence to indicate that bacteria can become *permanently* transformed from their basic structure or type, such as the production of bacilli from cocci or cocci from spirilla, under certain unfavorable conditions the form of a species may vary considerably from the true type. The atypical types may be *involution* or *degenerative* forms and are seen with certain organisms after prolonged growth upon artificial media, in media of unsuited reaction or under atmospheric conditions not conducive to good growth. Then, certain bacteria are designated as *pleomorphic* because, even as young cultures growing under the most favorable conditions, variation in form occurs. This is particularly true of Pfeifferella mallei, the causative agent of glanders. Long, slender rods showing a tendency towards branching, club-shaped forms and coccoid bodies are frequently seen in the same culture.

Flagella. It has been indicated that bacteria occur as motile and non-motile forms and, also, that when motile such property is due to flagella, except in the case of certain forms of spirochaetes which are motile by means of a type of undulating membrane. These flagella are hair-like appendages and, according to the species of organism, are found at one or both ends or all around the cell in numbers constant for the particular species. According to the number and location of the flagella bacteria are referred to as follows: *Monotricha* (single flagellum at one end), *amphitricha* (flagella at each end), *lophotricha* (several flagella at one end) and *peritricha* (flagella all around the organism). Non-flagellated bacteria are referred to as *atricha* organisms. Flagella cannot be detected by the ordinary methods of preparing specimens for microscopic examination. Special methods, as given under staining methods, are required for the demonstration of these appendages.

Capsules. Some organisms possess a mucin-like capsule which, in some cases at least, has a bearing on the virulence of the organism. For example, in the case of the anthrax bacillus, the more dense and less soluble its mucoid capsule the more virulent the organism.

Capsules are best demonstrated in material taken direct from the animal. In anthrax a blood film preparation from an animal dead of the disease affords excellent material for capsule demonstration. While special technique is required to bring out the capsule to advantage, the capsule "halo" can be frequently discerned even where the ordinary methods of staining are employed.

For the demonstration of capsules of organisms cultivated artficially best results are obtained where the growth has been in media containing milk, serum, or some albuminous material.

Spores. While bacteria multiply by *fission* (binary division) there are certain forms which produce *spores*, a form of resting stage of the bacterium which is capable of resisting unfavorable conditions. Thus, the types of bacteria most resistant to heat, drying, sunlight, chemicals, etc. are spore-bearers.

The *spore* is a highly refractile, round or oval body and occurs in or near the end of the organism in some species while in others it is central. An organism produces but one spore and this in turn but one bacillus. Thus, spore formation is not a method of multiplication of the organism but a means for its perpetuation. Atmospheric conditions play a part in the formation of spores by certain organisms. The anthrax organism, for example, can only form spores in the presence of free oxygen.

Metachromatic Granules. Certain bacteria possess granules which have a special affinity for the various dyes employed in bacteriology. When such bacteria are treated with a suitable dye the granules stand out as deeply stained areas. With polychrome stains these granules will frequently show a greater affinity for one color constituent of the dye than the remainder of the organism and are thus contrasted.

Composition of Bacterial Cell. Authorities do not agree on the exact structure of the bacterial cells. Aside from the flagella, capsules, and spores which certain forms possess, the basic cell is composed of a mass of protoplasm contained within a cell wall or membrane. There is considerable diversity of opinion as to the nature of the protoplasm. Some investigators maintain that it consists entirely of cytoplasm with no nuclear material whatever while others hold that it is all nucleus within a cell wall consisting of a dense cytoplasmic material. Another group maintains that the nucleus is separate and distinct from the cytoplasm proper, occurring essentially as it is found in higher cells. Still others hold that the nuclear material is distributed throughout the cytoplasm in the form of chromatin granules. The writer shares this latter view, believing that the bacterial cell consists of a definite cell wall composed of dense cytoplasmic material in which is contained a cytoplasm with an intermingling of nuclear material either in the form of granules of detectable size or in so finely divided a state as to make their differentiation exceedingly difficult or entirely impossible.

Chemically the bacterial cell consists largely of water, protein, fats, and salts of potassium, phosphorus, calcium, iron, sulphur and chlorine. The water content is ordinarily 80 to 90 per cent, the protein about 10 per cent, and the fat less than 1 per cent except in the case of a few organisms. These figures, obviously, will vary considerably with different types of bacteria.

REPRODUCTION, PHYSIOLOGY

Bacteria reproduce their kind by simple binary division or fission in the case of the non-sporulating forms while the spore-formers frequently have the spore stage intervening, in which case the spore germinates into the vegetative type (bacillus) and this in turn divides by fission, such process continuing until conditions become unfavorable when the bacilli cease dividing and form spores. Division of bacilli and spirilla is always at right angles to their long dimension. Where there is cohesion after division-bacilli are found in chains of varying lengths.

In the case of cocci the planes of division determine the grouping of the organism. Cocci which split in one plane and adhere form *streptococci*, in two planes *staphylococci*, in three planes *sarcina*. Adherence in pairs gives rise to *diplococci*. There is a marked cohesion with some forms while with others there is but little tendency in this direction.

Nutritional and Temperature Requirements. Ordinarily bacteria require for development media which will provide carbon, nitrogen, sulphur, phosphorus and salts of sodium or potassium and calcium or magnesium. Moisture in sufficient amount is a very necessary factor. Temperature is likewise an important factor, the requirements in this respect varying between wide limits with different organisms. Some bacteria (certain fungi, bacteria of sea-water, etc.) will actually propagate at 0°C. while at the other extreme we find forms (thermophiles) which will grow at temperatures between 65° and 70°C. While this wide temperature variation occurs with a few bacteria the majority of those which are pathogenic for man and animals have an optimum temperature requirement approximately equivalent to the body temperature of their host, in most cases around 37.5°C. Thus, for ordinary purposes the temperature of the bacteriological incubator is maintained at approximately 37.5°C.

Marked variation from the optimum temperature requirement of an organism for any considerable time, with certain forms of bacteria, has a detrimental effect not only on the growth of the organism but also on its virulence. Virulent anthrax bacilli, for example, when grown at a temperature of 42 to 43°C. for about twelve days, become so reduced in virulence that they will kill only small animals (mice and guinea pigs), causing but little disturbance in man and larger animals. If grown at such temperature for approximately twenty-four days the organism suffers a still greater loss of virulence being so reduced in such respect that it is only capable of producing disease in mice. Generally speaking, lower temperatures than those best suited to the growth of an organism, even though such tempera-

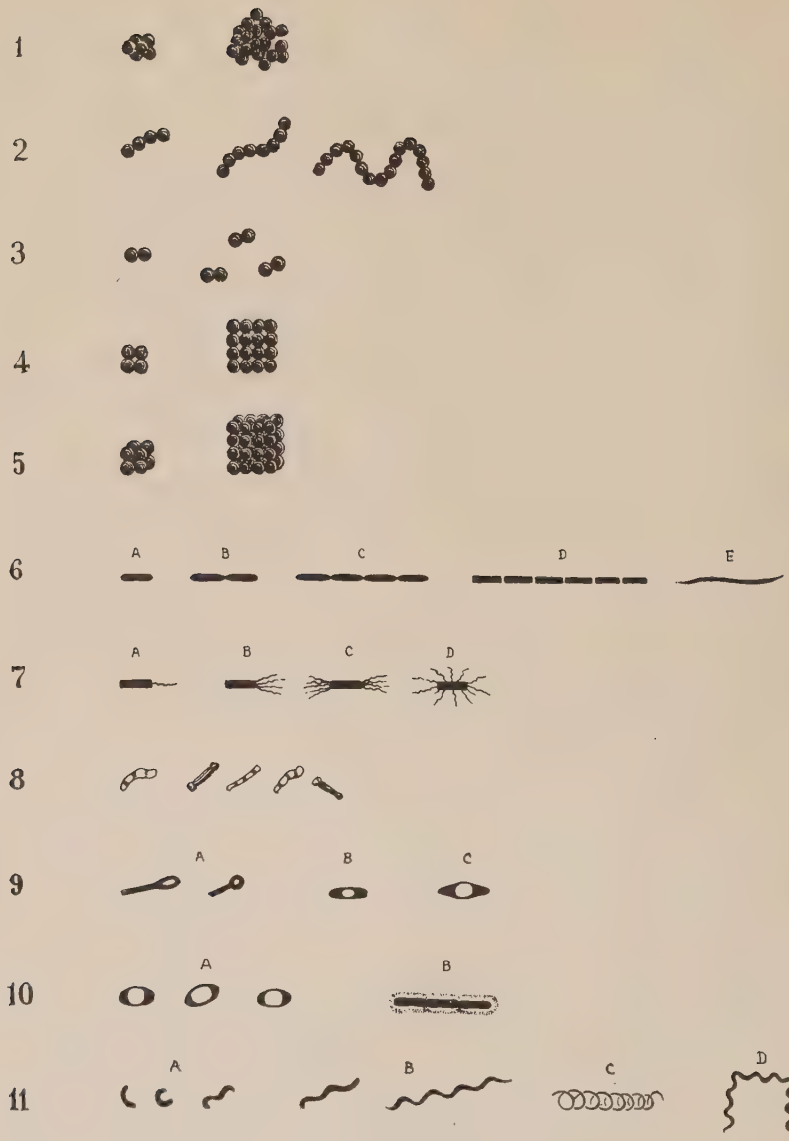


FIG. 1. DIAGRAMMATIC REPRESENTATION OF VARIOUS TYPES OF BACTERIA

1, staphylococcus; *2*, streptococcus; *3*, diplococcus; *4*, tetrads; *5*, sarcina; *6*, bacilli, (*a*) single bacillus, (*b*) bacilli in pairs, (*c*) chain of rounded end bacilli, (*d*) chain of bacilli with square-cut ends, (*e*) fusiform bacillus; *7*, flagellated bacilli, (*a*) monotricha, (*b*) lophotricha, (*c*) amphitricha, (*d*) peritricha; *8*, striated bacilli; *9*, sporulating bacilli, (*a*) terminal spores, (*b*) central spore, (*c*) clostridium; *10*, (*a*) bipolar-staining bacilli, (*b*) bacilli showing capsule; *11*, spirilla, (*a*) single curve, (*b*) several curves, (*c*) numerous curves, closely spiralled, cork-screw form, (*d*) numerous curves, loosely spiralled.

tures are extreme, exert no detrimental effect on an organism other than retarding its growth.

Atmospheric Requirements. Bacteria are divided into several main groups according to their growth in the presence or absence of oxygen. Those forms which require free oxygen for their growth are known as *strict* or *obligatory aerobes.* The second group consists of organisms which will propagate only when free oxygen is excluded and are known as *strict* or *obligatory anaerobes.* Intermediate between these groups we have *facultative anaerobes.* Such organisms will grow either in the presence or absence of oxygen. Most of them tend to grow best in the presence of oxygen, hence they are aerobes and facultative anaerobes. There are a few species of organisms which grow best in an atmosphere from which the oxygen is partially but not entirely removed. Such bacteria are *microaerophiles.*

The majority of pathogenic bacteria are aerobes and facultative anaerobes.

Reaction Requirements. Aside from the temperature and atmospheric environment, the reaction of the media in which bacteria are implanted for growth is an important factor. Some organisms require or grow best in media of varying acid reaction, others in alkaline media, and still others in media of a neutral reaction. The degree of acidity or alkalinity required varies with different species. With the majority of bacteria the extremes of the media reaction range are not so very far removed from the neutral point on the acid and alkaline sides. However, there are some types which require media of a rather highly acid reaction and others which require distinctly alkaline media.

Media Enrichments. In addition to the various requirements already mentioned, some species of bacteria will grow only sparsely or not at all unless certain special enrichening substances are present in the medium. Thus, one group of bacteria known as *hemoglobinophiles* will grow only when hemoglobin is included in the medium. In other cases blood serum, sugar, tissue fluids, etc., are essential to good growth.

Symbiosis; Commensalism. In connection with growth of bacteria we recognize that frequently two organisms growing together in the same culture or in the same body or lesion may be mutually helpful to one another's existence. Such condition is known as "symbiosis." Thus, it sometimes happens that an organism can grow and thrive in artificial culture in symbiosis with another organism under conditions which would not be conducive to its growth alone. Similarly, in the animal body it not infrequently happens that disease is produced where an organism is present symbiotically with another microbe, whereas the primary organism, in itself, might not have produced disease.

A parasitic organism which exists on the biproducts of another organism and does not produce disease is termed a "commensal."

Influences Detrimental to Bacteria. Drying, direct sunlight, putrefactive bacteria, bacteria producing various products of metabolism such as acid, lytic substances, etc., are detrimental to the life of many of the bacteria. Various chemical disinfectants and antiseptics are of course detrimental. Sera containing antibodies against certain organisms are, obviously, antagonistic to the bacteria for which they contain such specific antibodies. Plain water has a lytic effect on certain bacteria. Bile will kill others.

Bacteriophage. During the last few years considerable interest has been manifested in "bacteriophagy" or the "Twort-d'Hérelie phenomenon." As defined by d'Hérelle bacteriophagy is that phenomenon in which the dissolution of bacteria occurs through the operation of a principle which he terms "bacteriophage."

In 1915 Twort,[1] in working with filterable viruses, found on a plate culture containing colonies of micrococci, "watery-looking areas" in which the micrococci were split up. By placing a minute amount of material from one of these areas on a healthy, young colony on another plate, such colony was found to soon develop the "disease" and undergo the characteristic change.

Little attention was paid to Twort's observation until 1917 when d'Hérelle[2] started to report on the subject. As a result of his investigations, he concluded that the bacteriophage is an ultramicrobe which parasitizes bacteria, bringing about their destruction. He further concluded that the bacteriophage plays a major rôle in infection and immunity, constituting our chief defense against invading, pathogenic bacteria.

There has been considerable discussion as to the exact nature of this lytic agent called "bacteriophage." While some investigators agree with d'Hérelle's contention that it is a minute microbe living at the expense of bacteria, others are more inclined to believe that it is a type of chemical enzyme. Irrespective of what its true nature might be, there seems to be no doubt as to the ability of the agent to grow or multiply. It seems probable that it is a living structure.

Bacteriophage can be isolated from feces with but little difficulty. It can likewise be obtained from sewage without much trouble.

For a full discussion of bacteriophagy the reader is referred to "The Bacteriophage and Its Behavior," by d'Hérelle.[3]

[1] Twort, "An Investigation of the Nature of Ultramicroscopic Viruses," The Lancet, ii, 1241, 1915.

[2] d'Hérelle, Comp. rend. d l'Acad. Sci., clxv, 373, 1917.

[3] The Bacteriophage and Its Behavior, d'Hérelle, The Williams & Wilkins Co., Baltimore, Md.

Products of Bacterial Growth. In their metabolism bacteria produce various products such as gases, acids, pigments, enzymes, toxins, hemolysins, aggressins, etc. Some of these are produced in the body only, others only in culture, and still others both in the body and in culture.

The most common gas produced by bacteria is carbon dioxide. In addition nitrogen, oxygen, ammonia and hydrogen sulphide are produced in variable amounts by some bacteria. Of the acids formed by bacteria lactic acid is the most important. Other acids produced are acetic, butyric and formic.

A number of bacteria produce pigments of various colors. While such material apparently has nothing to do with the virulence of the organism it serves as an aid in identifying those species which produce it. Pigment production is usually facilitated by growing the particular organism at a temperature lower than that at which it normally grows best. Further, media which contain sulphur and magnesium compounds are conducive to pigment formation. The presence of free oxygen is necessary for pigmentation by most chromogenic bacteria while in a few instances the presence of free oxygen prevents the formation of pigment.

The more common enzymes produced by bacteria are: *proteases* which break up proteins with the formation of various split products such as proteoses, peptones, indol, skatol, amino-acids, and various gases; *carbohydrases* which split carbohydrates with formation of acid, gas, alcohol, etc.; *coagulase* which coagulates milk; and *lipases* which split fats producing fatty acids, glycerin, etc. In addition to these some bacteria have the property of producing *hemolysins* and other types of *cytolytic substances.*

Certain bacteria produce powerful toxins in and out of the body. In some cases this toxic substance is secreted by the bacterial cell while in other cases it is contained within the cell structure and is only liberated when the cell is split up. The first type is known as a *true* or *soluble toxin* while the latter is termed an *endotoxin.* These will be discussed more fully in a subsequent chapter.

Bacteria are capable of stimulating the production of antibodies such as opsonins, agglutinins, precipitins, bacteriolysins, complement-fixing bodies and various other types of immune bodies. These will be discussed in more detail in the chapter on immunity.

CLASSIFICATION OF BACTERIA

Numerous classifications and modifications of existing systems appear throughout the history of bacteriology. The earliest attempt at a systematic classification of bacteria of any note was by Mueller in 1773. In his

system he placed bacteria in the animal kingdom. In 1854 Cohn came out with the conclusion that bacteria should be placed in the vegetable rather than the animal kingdom. In 1857 Nageli published his classification in which he grouped bacteria in the plant kingdom under the class term "Schizomycetes." Since those early days numerous systems of classification and modifications of same have been published. Noteworthy are those of Cohn, Zopf, Flügge, Sternberg, Fischer, Migula, Lehmann and Newmann, Chester, Orla-Jensen, Buchanan, Winslow, and Bergey and his associates serving as a committee of the Society of American Bacteriologists for the classification of bacteria.

With the older methods of bacterial classification an organism's morphology was the all important factor in assigning it a proper place in the system. The short-comings of such a system have long been obvious to those interested in bacteriology. Under the system of classification adopted by the Society of American Bacteriologists, and followed in this book, it is not possible to merely study the microscopic appearance of an organism and then classify it. Before an organism can be classified under this system its morphologic, cultural and pathogenic properties must be determined. Thus, the name of an organism and its place in the system at once indicate something of these properties and do not merely suggest morphological characteristics.

The system starts by placing all forms of bacteria in the vegetable kingdom under the general class term "*Schizomycetes*" as first proposed by Nageli. This class term simply designates the various organisms as typically unicellular plants, usually small, of various shapes, and relatively primitive in organization.

Under the class are six *orders* as follows: (1) *Eubacteriales*, (2) *Actinomycetales*, (3) *Chlamydobacteriales*, (4) *Thiobacteriales*, (5) *Myxobacteriales*, and (6) *Spirochaetales*.

The first order (*Eubacteriales*) includes all of the simple and undifferentiated forms of bacteria—the true bacteria. This is the largest of the six orders. The other five orders include the various types of specialized or differentiated forms of bacteria. Thus the second order (*Actinomycetales*) deals with the plant- and mould-like types of the specialized or differentiated forms. In the third order (*Chlamydobacteriales*) sheathed organisms of the specialized or differentiated type which are not mould-like, are grouped. Order four (*Thiobacteriales*) includes unsheathed sulphur bacteria. The fifth order (*Myxobacteriales*) deals with slime-mould-like organisms, while the sixth order (*Spirochaetales*) includes protozoan-like forms. The names of orders are given the ending "*ales*."

Each order is next divided into one or more *families*. Under the order *Eubacteriales* we have five families as follows: (1) *Nitrobacteriaceae*, (2) *Coccaceae*, (3) *Spirillaceae*, (4) *Bacteriaceae*, and (5) *Bacillaceae*.

The first family (*Nitrobacteriaceae*) includes organisms of the *Eubacteriales* order which are obligate aerobes, using oxygen for direct oxidation of carbon, hydrogen, or nitrogen or compounds of same. The organisms composing this family are usually rod-shaped, occasionally round. The members of the four remaining families of this order do not receive their growth energy as the Nitrobacteriaceae. The second family (*Coccaceae*) includes the spherical forms. In the third family (*Spirillaceae*) are grouped spiral-shaped cells of the order. The fourth family (*Bacteriaceae*) includes motile and non-motile straight rods which do not produce endospores. The fifth family of the order (*Bacillaceae*) deals with members which usually produce endospores.

The order *Actinomycetales* has at present but two families under it, namely, *Actinomycetaceae* and *Mycobacteriaceae*. The members of the first family (*Actinomycetaceae*) are filamentous forms, frequently found branching and only occasionally parasitic. The forms falling in the second family (*Mycobacteriaceae*) are parasitic, rod-shaped, rarely filamentous and only occasionally found branching.

The order *Chlamydobacteriales* contains only one family—*Chlamydobacteriaceae*. This family includes alga-like, filamentous bacteria, typically water forms, frequently sheathed, no true branching.

There are three families under the order *Thiobacteriales*. They are: (1) *Rhodobacteriaceae*, (2) *Beggiatoaceae*, and (3) *Achromatiaceae*. The first family includes cells containing bacteriopurpurin with or without sulphur granules. The second and third families are both made up of cells regularly containing sulphur granules but no bacteriopurpurin. The difference between the second and third families is found in the form of the organisms. Those in the second family are filamentous while the members of the third family are motile, unicellular forms, not filamentous.

The fifth order *Myxobacteriales* contains but one family—*Myxbacteriaceae*. This family includes a group of motile, rod-shaped organisms which multiply by fission and which secret a gelatinous-like base.

The order *Spirochaetales* also has but one family under it, the *Spirochaetaceae*. This group includes protozoan-like, relatively slender, flexous spirals, frequently multiplying by longitudinal division.

The family names designate the name of one of their principal genera with the ending "*aceae*."

To continue the classification three families (*Nitrobacteriaceae*, *Coccaceae*,

TABLE 1

Schizomycetes

ORDER	FAMILY	SUBFAMILY	TRIBE	GENUS	TYPE SPECIES
I. Eubacteriales	I. Nitrobacteriaceae		I. Nitrobacterieae	I. Hydrogenomonas	Hydrogenomonas bantotropha
				II. Methanomonas	Methanomonas-methanica
				III. Carboxydomonas	Carboxydomonas oligocarbophila
				IV. Nitrosomonas	Nitrosomonas europaea
				V. Nitrobacter	Nitrobacter winogradskyi
				VI. Nitrosococcus	Nitrosococcus nitrosus
				VII. Acetobacter	Acetobacter pasteurianum
				VIII. Thiobacillus	Thiobacillus thioparus
			II. Azotobacterieae	IX. Azotobacter	Azotobacter chroococcum
				X. Rhizobium	Rhizobium leguminosarum
	II. Coccaceae		I. Streptococceae	I. Diplococcus	Diplococcus pneumoniae
				II. Streptococcus	Streptococcus pyogenes
				III. Leuconostoc	Leuconostoc mesenteroides
			II. Neisserieae	IV. Neisseria	Neisseria gonorrhoeae
				V. Gaffkya	Gaffkya tetragena
			III. Micrococceae	VI. Staphylococcus	Staphylococcus aureus
				VII. Micrococcus	Micrococcus luteus
				VIII. Sarcina	Sarcina ventriculi
				IX. Rhodococcus	Rhodococcus rhodochrous
	III. Spirillaceae			I. Vibrio	Vibrio comma
				II. Spirillum	Spirillum undula
	IV. Bacteriaceae		I. Chromobacterieae	I. Serratia	Serratia marcescenes
				II. Flavobacterium	Flavobacterium aquatilis
				III. Chromobacterium	Chromobacterium violaceum
				IV. Pseudomonas	Pseudomonas aeruginosa
			II. Cellulomonadeae	V. Cellulomonas	Cellulomonas biazotea

I. Eubacteriales	IV. Bacteriaceae		III. Achromobacterieae	VI. Achromobacter	Achromobacter liquefaciens
			IV. Lactobacilleae	VII. Lactobacillus	Lactobacillus caucasicus
			V. Erwinieae	VIII. Erwinia	Erwinia amylovora
				IX. Phytomonas	Phytomonas campestris
			VI. Kurthieae	X. Kurthia	Kurthia zopfii
			VII. Bacterieae	XI. Escherichia	Escherichia coli
				XII. Aerobacter	Aerobacter aerogenes
				XIII. Proteus	Proteus vulgaris
				XIV. Salmonella	Salmonella suipestifer
				XV. Eberthella	Eberthella typhi
				XVI. Alcaligines	Alcaligines fecalis
			VIII. Pasteurelleae	XVII. Pasteurella	Pasteurella avicida
			IX. Klebsielleae	XVIII. Klebsiella	Klebsiella pneumoniae
			X. Hemophileae	XIX. Hemophilus	Hemophilus influenzae
				XX. Dialister	Dialister pneumosintes
			XI. Bacteroideae	XXI. Bacteroides	Bacteroides fragilis
	V. Bacillaceae			I. Bacillus	Bacillus subtilis
				II. Clostridium	Clostridium butyricum
II. Actinomycetales	I. Actinomycetaceae			I. Actinobacillus	Actinobacillus lignieresi
				II. Leptotrichia	Leptotrichia buccalis
				III. Actinomyces	Actinomyces bovis
				IV. Erysipelothrix	Erysipelothrix rhusiopathiae
	II. Mycobacteriaceae			I. Mycobacterium	Mycobacterium tuberculosis.
				II. Corynebacterium	Corynebacterium diphtheriae
				III. Fusiformis	Fusiformis termitidis
				IV. Pfeifferella	Pfeifferella mallei

ORDER	FAMILY	SUBFAMILY	TRIBE	GENUS	TYPE SPECIES
III. Chlamydobacteriales	I. Chlamydobacteriaceae.			I. Leptothrix	Leptothrix ochracea
				II. Didymohelix	Didymohelix ferruginea
				III. Chrenothrix	Chrenothrix polyspora
				IV. Sphaerotilus	Sphaerotilus natans
				V. Clonothrix	Clonothrix fusca
IV Thiobacteriales	I. Rhodobacteriaceae.	I. Chromatoideae	I. Thiocapseae	I. Thiocystis	Thiocystis violacea
				II. Thiosphaera	Thiosphaera gelatinosa
				III. Thiosphaerion	Thiosphaerion violaceum
				IV. Thiocapsa	Thiocapsa roseopersicina
				V. Thiosarcina	Thiosarcina rosea
			II. Lamprocysteae	I. Lamprocystis	Lamprocystis roseoperscina
			III. Thiopedieae	I. Thiopedia	Thiopedia rosea
				II. Thioderma	Thioderma rosea
				III. Lampropedia	Lampropedia hyalina
			IV. Amoebobacterieae	I. Amoebobacter	Amoebobacter roseum
				II. Thiodictyon	Thiodictyon elegans
				III. Thiothece	Thiothece gelatinosa
				IV. Thiopolycoccus	Thiopolycoccus ruber
			V. Chromatieae	I. Chromatium	Chromatium okenii
				II. Rhabdomonas	Rhabdomonas rosea
				III. Thiospirillum	Thiospirillum sanguineum
				IV. Rhodocapsa	Rhodocapsa suspensa
				V. Rhodothece	Rhodothece pendens
		II. Rhodobacterioideae		I. Rhodocystis	Rhodocystis gelatinosü
				II. Rhodonostoc	Rhodonostoc capsulatus
				III. Rhodorhagus	Rhodorhagus capsulatus
				IV. Rhodobacterium	Rhodobacterium capsulatum
				V. Rhodobacillus	Rhodobacillus palustris
				VI. Rhodovibrio	Rhodovibrio parvus
				VII Rhodospirillum	Rhodospirillum rubrum

IV. Thiobacteriales	II. Beggiatoaceae			I. Thiothrix	Thiothrix nivea
				II. Beggiatoa	Beggiatoa alba
				III. Thioploca	Thioploca schmideii
	III. Achromatiaceae			I. Achromatium	Achromatium oxaliferum
				II. Thiophysa	Thiophysa volutans
				III. Thiospira	Thiospira winogradskyi
				IV. Hillhousia	Hillhousia mirabilis
V. Myxobacteriales	I. Myxobacteriaceae			I. Myxococcus	Myxococcus rubescens
				II. Polyangium	Polyangium vitellinum
				III. Chondromyces	Chondromyces crocatus
VI. Spirochaetales	I. Spirochaetaceae			I. Spirochaeta	Spirochaeta plicatilis
				II. Saprospira	Saprospira grandis
				III. Cristispira	Cristispira balbiani
				IV. Borrelia	Borrelia gallinarum
				V. Treponema	Treponema pallidum
				VI. Leptospira	Leptospira ictohemorrhagica

Bacteriaceae) of the first order (*Eubacteriales*) are divided into *tribes* and the tribes into *genera*. The families *Spirillaceae* and *Bacillaceae* of this order are divided into genera without intervening tribes. Of the other five orders, four (*Actinomycetales, Chlamydobacteriales, Myxobacteriales and Spirochaetales*) are divided into genera from families, no sub-families or tribes being included. The first family (*Rhodobacteriaceae*) of the order *Thiobacteriales* is divided first into *sub-families*, then into tribes and genera. The remaining two families of this order are divided into genera direct from families.

To complete the system all of the various genera are then divided into *species*. The species, of course, are made up of the different *strains* of like organisms.

The names of sub-families are taken from the names of the genera in the group and are given the ending "*oideae*." Tribes are named in the same way except that the ending given the generic name selected is "*eae*." Generic names, comparable with our own family names, may be names of persons, may be arbitrarily composed, or taken from any source whatever. In giving a specific name it is aimed to give some indication of the appearance, characters, origin, history or properties of the species. It is permissible, however, to use the name of the person who discovered or described the organism as the specific name.

To recapitulate; according to its morphologic, cultural and pathogenic characteristics, an organism is first classified under an order of Schizomycetes; next it is placed in a family under the particular order; then in some cases in a sub-family and tribe; next in a genus, and finally is classified as a species of the particular genus.

Table 1 depicts the system of classification advocated by the Committee of the Society of American Bacteriologists; the system adopted in grouping the bacteria dealt with in this book.

PART II

BACTERIOLOGICAL METHODS

CHAPTER III

The Microscope

A thorough understanding of the microscope and its use is a prerequisite to the study of bacteriology. Too frequently the student is satisfied with a bare working knowledge of the instrument. This can only result in a real handicap as, obviously, the more accurate definition, etc., one can obtain with his microscope the better bacteriology he is capable of doing. A little more time spent in the beginning, becoming well acquainted with the instrument, will later result in more satisfactory work.

The compound microscope ordinarily used in bacteriological work consists of the following parts:

Base
Stage
Arm
Tube
Draw-tube
Eye-piece or ocular
Nose-piece (for 2, 3, or 4 objectives)
Objectives
Coarse adjustment screw
Fine adjustment screw
Substage which carries { Condenser / Iris diaphragm
Double mirror (plane and concave)

Objectives. These are the mounted lenses of the microscope and constitute the most important pieces in its make-up. There are three objectives commonly employed in bacteriological work. Two of these objectives carry dry lenses while the third has an oil immersion lens. Objectives are usually designated by their *equivalent focal* distances. The two objectives carrying the dry lenses usually have equivalent focal distances of 16 and 4 mm. respectively. The objective carrying the immersion lens ordinarily has an equivalent focal distance of 1.9 or 2 mm.

In order to explain equivalent focal distance it is first necessary to define principal focal distance. When rays of light enter a converging lens parallel to the optic axis the distance from the center of the lens to the point where such rays, after passing through the lens, cross the axis is known as the

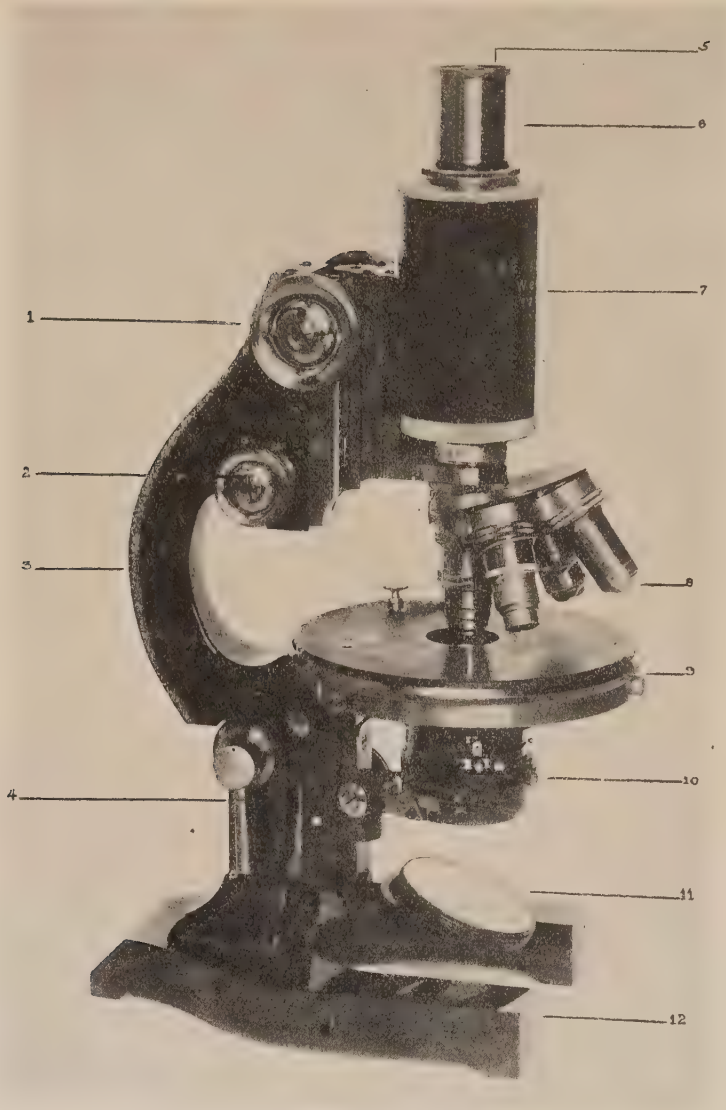


FIG. 2. COMPOUND MICROSCOPE

1, coarse adjustment screw; *2*, fine adjustment screw; *3*, arm; *4*, adjustment lever used in tilting microscope; *5*, eye-piece or ocular; *6*, draw-tube; *7*, tube; *8*, four objectives screwed in nose-piece; *9*, stage; 10, substage, carrying condenser and iris diaphragm; *11*, mirror—one side plane the other concave; *12*, base.

principal focal distance. Now take, for purposes of illustration, an objective designated as having an equivalent focal distance of 4 mm. or $\frac{1}{6}$ inch. Such designation merely means that the objective produces a real image of the same size as that produced by a *simple converging lens* whose principal focal distance is 4 mm. or $\frac{1}{6}$ inch. As the relative size of object and image vary in direct proportion to their variation in distance from the center of the lens the less the equivalent focal distance the greater the size of the image. This is readily apparent when it is recalled that the tube length of the microscope remains constant (usually 160 mm.).

The equivalent focal distance does not represent the actual working distance of the objective, i.e., distance between top of cover slip and lower lens of the objective. In other words when an object is in exact focus, say with the 4 mm. objective, the actual distance between lens and cover slip may be only 2.50 mm. or even less.

TABLE 2

Microscope

Magnification table

OBJECTIVE	OCULAR				
	5 ×	6.4 ×	10 ×	12 ×	20 ×
mm.					
16	50	64	100	120	200
4	215	275	430	518	860
1.9	475	608	950	1040	1900

Parfocal Objectives. The best microscopes have the objectives so constructed and arranged in the triple nose piece that they are approximately *parfocal.* This means that when one objective is adjusted so that the object is in focus any one of the other objectives can be swung in the focal axis and such objective will be found to have its lenses in approximate focus.

Objective Magnification. The 16, 4 and 1.9 mm. objectives in themselves give magnifications of about 10, 43 and 95 diameters respectively. This primary magnification is further increased according to the power of the ocular or eye-piece used.

Oculars. The ocular or eye-piece contains lenses for the purpose of magnifying the image formed by the objective. Oculars are usually numbered according to the magnification of which they are capable. Thus a 6.4 ocular magnifies the objective image 6.4 times, a 10 ocular ten times, etc.

The 6.4 × and 10 × are the oculars most commonly employed but oculars may be obtained in a range of from 2 × to 20 ×.

Table 2 gives the magnifications obtained with the various combinations of objectives and oculars commonly employed.

Condenser. As the term implies the condenser serves to condense the reflected light so that the microscopic field is amply illuminated. The condenser consists of a system of lenses, the function of which is to converse the reflected rays so that they are evenly concentrated in the microscopic field.

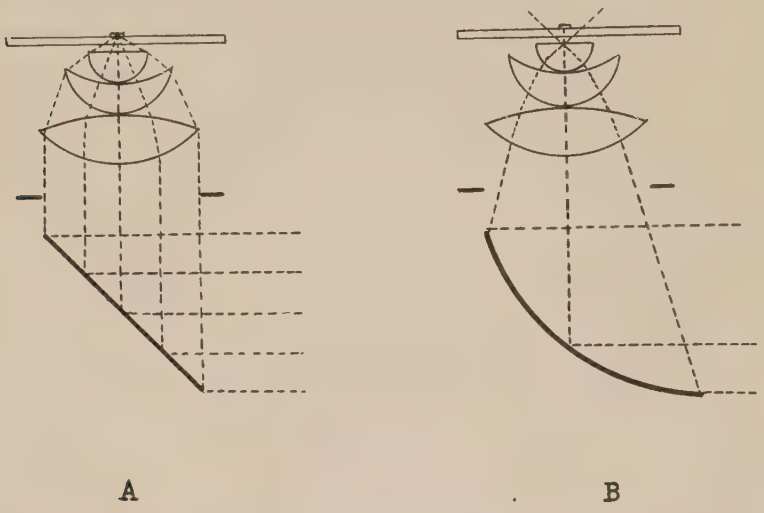


FIG. 3. DIAGRAM ILLUSTRATING PATH OF LIGHT RAYS THROUGH CONDENSER (*a*) with plane mirror, (*b*) with concave mirror

Mirrors. In using the condenser the plane mirror should invariably be employed. If the concave mirror is used the light rays converge below the uppermost lens of the condenser with the result that the apex of the cone of light is within the condenser rather than in the plane of the object.

Tube Length. The tube length is the distance from the upper lens of the ocular to the distal end of the tube at the point where the objective is screwed in. Most objectives are corrected for use with a tube length of 160 mm. As the thickness of cover slip figures in the computation those of 0.18 mm. thickness are usually specified. Thinner cover slips, however, can be employed with satisfactory results.

Numerical Aperture. The term "numerical aperture," abbreviated "N. A.," and commonly found marked on microscope objectives, was first

introduced by Professor Abbe to indicate the "capacity for receiving rays from the object and transmitting them to the image." In a general way the N. A., as applied to microscope objectives, indicates the relative proportion of light rays, passing from the microscopic field, which can enter the objective and form the image. The greater the numerical aperture of an objective the more light it will admit, which, of course, makes for better definition.

Homogeneous Oil Immersion Lens. With the dry lenses air is interposed between the cover glass and lower lens of the objective. As a result of the difference between the refractive index of air and glass, part of the light rays are deflected and do not enter the lens of the objective. When the homogeneous oil immersion lens is employed a drop of cedar oil, having the same refractive index as glass, is placed on the coverslip and the lens immersed in the oil. Thus, with no air interposed the refractive index is the same throughout and a greater percentage of the light rays enter the objective.

Dark Field Illumination. For the examination of living bacteria, certain protozoan organisms, etc., certain advantages are obtained from a dark ground examination. In such examinations the objects to be studied appear as brightly illuminated structures in a dark field.

The dark field illuminator, which is placed in the microscope in place of the ordinary condenser, is so constructed that when properly adjusted only very oblique rays of light strike the object and no direct light enters the objectives.

If the N. A. of the objective to be employed for the darkfield examination is not 1.0 or less a funnel-stop must be used to reduce the numerical aperture, otherwise too much light will enter the lens and prevent the production of a dark ground. The funnel-stop is placed in the lower portion of the objective. Some types are made to screw in, others are merely dropped into place.

Before a satisfactory dark field examination can be made there are certain basic factors which must be taken into consideration. First, the dark ground illuminator must be properly centered. This is accomplished by focusing with the low-power objective until the ring inscribed on the illuminator comes into view. Then the adjusting screws of the condenser are manipulated until the ring is exactly in the center of the field. Next, a very strong light is required for dark field work. An arc light can be used or any one of several types of lamps on the market for the purpose may be employed. A reflector is used so that the rays of light are brought to a cone on the mirror.

It is highly important that thin slides and cover-slips be used. If they are too thick they are worthless for dark field work. It is also important

that the preparation of the specimen to be examined be carefully carried out. Heavy suspensions are unsatisfactory.

Use of the Microscope. The microscope should be used on a table where it will receive the very minimum of vibration. When daylight is employed for illumination of the field a north light is preferable. After the microscope is placed the low-power dry objective is thrown into the focal axis and the plane mirror manipulated while looking into the instrument until the field appears brightly and evenly illuminated.

When preparations are to be examined the slide is placed on the stage and the objective lowered almost to the slide, then looking into the microscope the instrument is focused up. Never focus down as the lens and slide may both be broken.

In using the microscope it is good practice to acquire the habit of using both eyes. Continual use of one eye causes strain. Keep the eye not in use open. When lengthy examinations are conducted the binocular microscope is preferable to the single ocular scope. It is less satisfactory, however, for fine definition.

Always make sure the draw tube is properly adjusted.

After using the oil immersion lens clean the same with Japanese lens paper before putting away. If oil is forgotten and it gets thick on the lens it may be removed with a little xylol on lens paper. Always dry with clean paper after using xylol, however, as it may soften the balsam with which the lens is cemented into the objective.

Protect the microscope from heat, sun, and dust. When not in use keep it in its case or under a bell jar.

See that the lens of the oculars and the condenser, as well as those of the objectives, are kept clean. Keep the mirror clean.

CHAPTER IV

Sterilization, Preparation of Glassware, etc.

The various methods of sterilization utilized in the manufacture of culture media, in the preparation of laboratory apparatus, and for the destructon of bacteria in discarded cultures, specimens, etc., are of basic importance. Sterile culture media, glassware, etc., plus careful technique on the part of the student are basic requirements and very essential if contamination, the bogey of bacteriology, is to be made a negative factor.

Bacteria may be destroyed in a number of ways. Many types, if merely deprived of moisture, soon die. Some forms, however, can withstand drying for long periods of time. Direct sunlight is a powerful disinfectant destroying even the most resistant forms of bacteria if given sufficient time.

Aside from the natural factors which are detrimental to the well-being of bacteria, there are a number of methods available to the bacteriologist for the destruction or elimination of microörganisms. The choice of the method depends, on one hand, upon the resistance of the organism or organisms, when dealing with known species, and, on the other hand, with the nature of the material to be sterilized. Some bacteria are very easily destroyed while others are extremely resistant. Thus, one method of sterilization may be perfectly satisfactory for the destruction of one type of organism but absolutely incapable of destroying a more resistant type. Then, when dealing with certain types of culture media, biological products, etc., the effect of the different methods of sterilization on the media or biologic must be considered in choosing the method. Thus, in some cases heat sterilization cannot be applied without ruining the product. Filtration can be resorted to for sterilization in some such cases. Then when it comes to sterilizing the hands, tissue surfaces, etc., chemical agents are employed. The methods of sterilization commonly employed in bacteriological work may be grouped as follows:

- Heat
  - Dry
    - Direct flame
    - Hot air
  - Moist
    - Boiling water
    - Flowing steam
    - Steam under pressure

Filtration { Through the use of filters such as Berkefeld, Chamberland, Pasteur, etc.

Chemical { Various chemical bactericides including the gases used in fumigation

Sterilization by means of heat is the most common and the most important of the methods ordinarily employed for the destruction of bacteria. The efficacy of heat, however, is directly dependent upon its method of application.

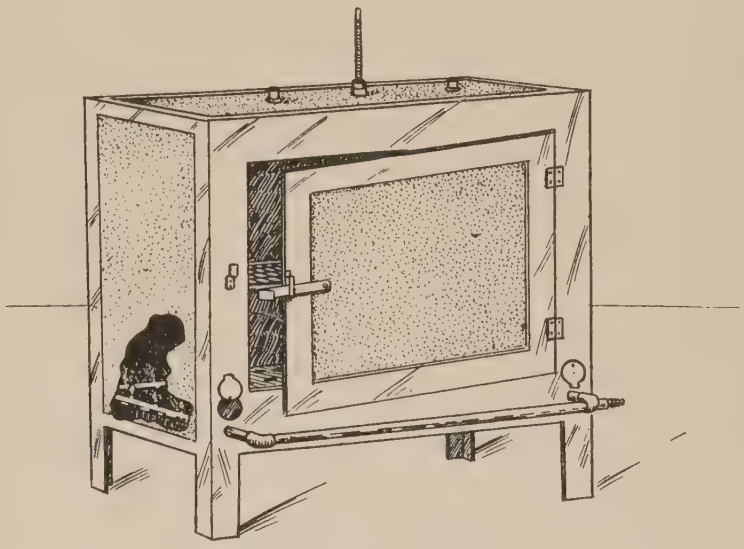


Fig. 4. Hot Air Sterilizer

Incineration. Incineration or sterilization by heating an object in *direct flame* is, obviously, sure to result in the complete destruction of all forms of bacteria. This method is, of course, limited in its application, being used only for the sterilization of such things as platinum loops, spatulas, needles, etc., and for the disposal of diseased tissues, waste, etc.

Hot Air Sterilization. For the sterilization of glassware and such other laboratory apparatus as it is desired to have perfectly dry during and following sterilization *hot air* is employed. A hot air sterilizer (Fig. 4) maintained at a temperature of about 160°C. for one hour, is employed for this type of sterilization. Such sterilizers are usually heated by gas although electrically heated and controlled ovens can be obtained. The

latter are slow in "coming up" and besides are quite expensive. It is difficult to operate hot air ovens satisfactorily with kerosene burners.

In sterilizing glassware the same should be placed in the oven and the temperature slowly brought up to 160°C., maintained at such for one hour and the heat shut off. The door of the oven should not be opened until the temperature has dropped to 50°C. or less, otherwise glassware is likely to crack.

Sterilization by Boiling. *Moist heat* can be applied in the form of boiling water, flowing steam or steam under pressure. Sterilization by *boiling in water* is a simple and useful everyday procedure. Surgical instruments, hypodermic syringes, etc., are commonly sterilized in this manner. While the method is of great practical value and sufficiently satisfactory for a great many purposes, the spores of some of the more resistant bacteria are able to tolerate the action of boiling water for long periods of time.

Flowing Steam Sterilization. Sterilization by *flowing steam* (fractional sterilization) is accomplished through the use of the *Arnold Sterilizer* (Fig. 5). The material to be sterilized is placed in the Arnold and subjected to the action of the flowing steam at 100°C. for one-half hour. The process is repeated on the second and third day following. The first exposure kills all vegetative forms of bacteria. The material is then permitted to stand at room or incubator temperature until the next day during which time most of the spores which resisted the first sterilization will have germinated. The second sterilization will then destroy those. By allowing another twenty-four hours standing or incubation any spores which may not have germinated between the first and second sterilizations will, in the vast percentage of cases, then have germinated and will be destroyed by the third sterilization. Care must be taken not to start to time the half-hour period until the temperature reaches its maximum (approximately 100°C. varying slightly with altitude).

The fractional method of sterilization is of particular value in the sterilization of culture media containing carbohydrates, gelatin, milk, etc., which would be altered in character by high temperatures.

The original type of Arnold sterilizer was of a cylindrical type, the outer jacket being constructed to lift off when putting material in or taking it out of the sterilizer. This same type is used by a large number of laboratories. The square type with door (Boston Board of Health type) is more desirable.

Sterilization by Steam Under Pressure. This means of sterilization constitutes the most efficient method we have. The apparatus used is known as the *Autoclave* (Fig. 7). Various kinds of autoclaves, of both vertical and horizontal types, are on the market, The steam can be generated by means

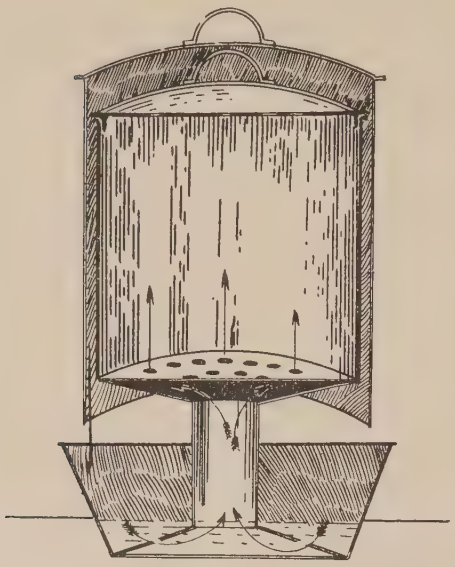


FIG. 5. ARNOLD STERILIZER; CYLINDRICAL TYPE

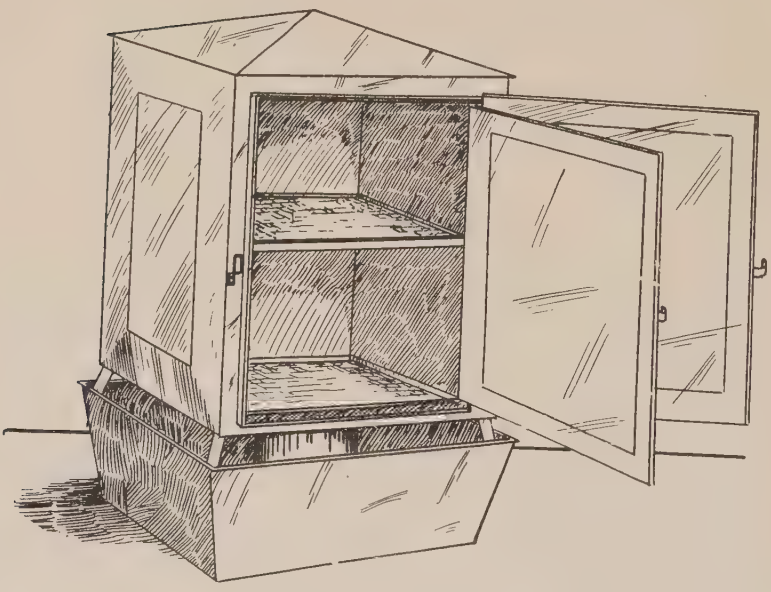


FIG. 6. ARNOLD STERILIZER; BOSTON BOARD OF HEALTH TYPE

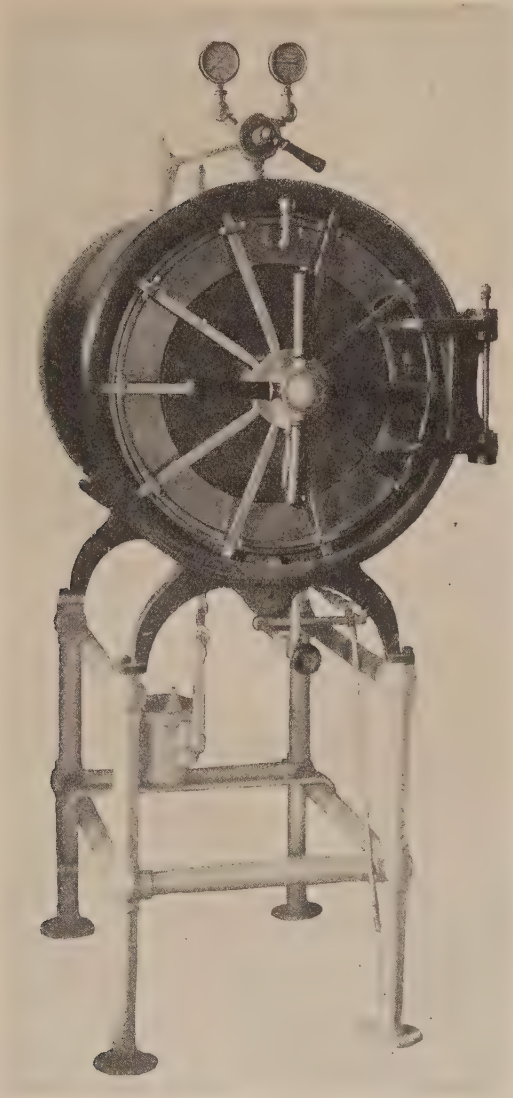


FIG. 7. AUTOCLAVE

of a boiler furnished as a part of the apparatus and heated by gas, kerosene, gasoline or alcohol, or where steam from a central plant is available, the boiler can be dispensed with and the steam supplied direct, thus saving considerable time in operating the sterilizer.

For most purposes the autoclave is operated under steam pressure of 15 pounds. Such pressure gives a temperature of about 121°C. which, when maintained for one-half hour, is sufficient to destroy the most resistant forms of bacteria. Some laboratories, however, operate their autoclave at different pressures ranging from 10 to 20 pounds. The temperatures obtained with different pressures are given in Table 3.

In operating the autoclave it is highly important that all of the air be exhausted before closing the valve, otherwise the temperature of the material or article to be sterilized may be less than that which the pressure would indicate and, hence, might not be effectively sterilized. Do not permit the autoclave pressure to rise too rapidly when starting the sterilizer. This

TABLE 3

PRESSURE	TEMPERATURE
pounds	*°C.*
5	108.80
10	115.60
11	116.80
12	118.00
13	119.10
14	120.20
15	121.30
16	122.40
17	123.30
18	124.30
19	125.40
20	126.20

applies particularly where a direct steam supply is used. A too rapid rise may result in breakage. Likewise, do not permit the pressure to drop suddenly. If this is done stoppers blow out of flasks and bottles or are wet by the fluid bubbling up, and, in the case of fragile glassware, a sudden drop in pressure will result in a high percentage of breakage.

In addition to its use in the sterilization of various laboratory apparatus, media, equipment, reagents, etc., the autoclave is the proper sterilizer for rendering safe all discarded cultures, infectious material, etc., and should, as a matter of practice, be utilized for such purpose in preference to other methods.

Sterilization by Filtration. Filtration through bacteria-retaining filters constitutes a means for the sterilization of various types of culture media, biological products, etc., which cannot be rendered sterile by the applica-

tion of heat. Culture media containing serum or carbohydrates, which would be spoiled by heat can be sterilized by filtration without spoiling the product or changing its character. Filtration sterilization is employed extensively in the commercial manufacture of biological products. It is an

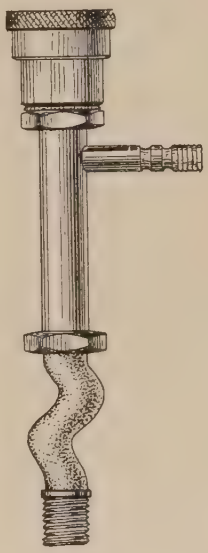


Fig. 8 Fig. 9

Fig. 8. Berkefeld Filter and Filtering Flask

Fig. 9. Richard's Vacuum Pump

indispensable method for the sterilization of various antitoxins, antisera, tuberculin, mallein, aggressins, etc.

Several types of bacteria-retaining filters are available for bacteriological work. Those most commonly employed are the Berkefeld, Pasteur and Chamberland. Of these the Berkefeld is most used. These filters come in the form of hollow candles or bougies of diatomaceous earth in the case of

the Berkefelds and unglazed porcelain in the case of the Pasteur and Chamberland filters. The open end of the cylinder is cemented into a metal or porcelain head-piece which suffices for the connection of the filter with the flask or bottle receiving the filtrate. Filters may be obtained in a variety of sizes and in different degrees of porosity. In the case of the Berkefeld they are designated as follows:

V = fine
N = normal (medium)
W = coarse

Filtration with these filters is accomplished by means of suction or pressure. For ordinary laboratory work suction obtained by means of a Richards filter pump (Fig. 9), attached to a water faucet, will usually suffice. Where heavy material, such as globulin solutions (antitoxins), aggressins, etc., are to be filtered on a large scale, and where time is a factor, as in the commercial manufacture of biologics, pressure filtration should be resorted to.

Obviously, it is most important in this type of sterilization that the filters be sound, the apparatus sterile, and that there are no air leaks at any of the connections, particularly where suction is used. All filters should be tested when first obtained and at occasional intervals afterwards, by passing a bouillon culture of Serratia marcescens (B. prodigiosis) through the filter and incubating the filtrate. If the filtrate becomes cloudy it can be subcultured on solid medium and the organism readily identified by the red pigment produced.

Before using new filters they should be thoroughly washed by passing an abundance of distilled water through them. After they have been used filters should be thoroughly cleaned. If utilized for the filtration of infectious material they should first be sterilized by boiling or autoclaving and then cleaned. If coagulable material has been filtered it is necessary that the filter be well washed by passing plenty of water through it before sterilizing. Filter candles can be cleaned by first scrubbing their surface well with a brush, following this by boiling in a 5 per cent solution of NaOH, and finally washing them well by passing distilled water through them. The following method also gives good results. After scrubbing the surface, a 0.5 per cent solution of potassium permanganate can be put through them, followed by a 5 per cent solution of sodium bisulphite, and then by a good quantity of distilled water.

After filters have been cleaned they should be attached to the suction pump to draw as much of the water out of them as possible and then they

should be dried out well by sterilizing in the hot air oven. They should never be put away damp. If they are not dry moulds will frequently grow on their surfaces and if not removed promptly may, in a short time, penetrate the filter substance and sometimes destroy its bacteria-retaining efficacy.

In setting up a filtering apparatus the entire set up can be made and the assembled apparatus sterilized in the autoclave just prior to use. Or, if desired, the flasks and filters can be sterilized in the hot air sterilizer and kept on hand ready for use, the rubber connections being sterilized by prolonged boiling or autoclaving just previous to assembling the apparatus. The writer makes it a practice to maintain the filters and flasks on hand sterilized and, just prior to use, rubber connections are boiled in water for one-half hour, the apparatus assembled and then, as an additional precaution against the possibility of contamination, the entire equipment is autoclaved at 15 pounds pressure for one-half hour. Before starting to filter, all joints in the system should be sealed by the application of melted paraffine.

Where fluids containing considerable suspended material are to be filtered, it is frequently necessary to first pass the fluid through paper pulp or asbestos wool. This is accomplished by making a mat of paper pulp or fine asbestos wool over the perforated plate of a Buchner funnel, placing the funnel in a filtering flask and drawing the fluid through by means of suction. The thickness of the mat should be regulated according to the nature of the material to be filtered, making the mat just sufficiently thick to clear the fluid so it will readily pass through the bacteria-retaining filter. If the mat is made too thick the process is considerably delayed.

Paper pulp is prepared by macerating small torn bits of filter paper in water. Asbestos fiber prepared especially for filtering can be obtained from dealers in chemicals and laboratory materials.

It is sometimes advisable to filter first through a coarse filter and then through one of finer porosity. This will frequently facilitate the filtration of certain fluids. Then, where it is desired to make sure that sterilization has been accomplished, in cases where the product will not be impaired by repeated filtration it can be put through several normal or fine filters rather than a single one.

Chemical Sterilization. The addition of such chemical agents as phenol, mercuric chloride, formalin, iodine, chlorine, etc., in sufficient concentration, will destroy bacterial life. Then, certain gases liberated and confined to a given atmosphere are of value. Such process is commonly termed "*fumigation.*" These methods of sterilization or disinfection are highly impor-

tant, frequently being the only means we have for the sterilization of certain things. The sterilization of the hands, surfaces of the body, various types of equipment, premises, water, waste, etc., bring chemical agents into prominence. In the preservation of antitoxins, sera, and various other biological products, phenol, "Trikresol," chloroform, etc., are extensively employed.

The chemical disinfectants very commonly found on the shelf of the average laboratory are phenol in 5 per cent solution, liquor cresolis compound in 3.5 per cent solution, and bichloride of mercury in a dilution of 1 to 1000. In addition of course, tincture of iodine, U. S. P., is always in evidence.

The gas most commonly employed for fumigation is formaldehyde. There are several methods of generating it for such purpose. The method most commonly employed is the potassium permanganate method. One part of potassium permanganate is used with two parts of formalin. The permanganate is placed in a bucket or container sufficiently deep to take care of the slight amount of foaming which occurs during the reaction. After the room or other compartment has been made as airtight as possible the receptacle containing the permanganate of potash is placed in the room and the proper proportion of formalin poured on same. The operator quickly retires, closing the door and properly sealing it. After 24 hours the room can be opened and ventilated. Two hundred fifty grams of potassium permanganate and 500 cc. of formalin are required for every 1000 cubic feet.

Another method of generating the gas is by adding formalin to CaO (quicklime). Where this method is employed 10 per cent concentrated sulphuric acid is added to the formalin and 500 cc. of this mixture is employed with 800 grams of quicklime for every 1000 cubic feet of air space.

In addition to the above methods there are several types of formaldehyde gas generators on the market which give very satisfactory results in fumigation.

PREPARATION OF GLASSWARE, ETC.

All glassware employed in the laboratory should be kept scrupulously clean. Even with the best of care it is sometimes impossible to prevent test tubes, flasks, etc. from becoming "smoky," especially when poor grades of glass have been used in the making of the apparatus.

Cleaning Glassware. Ordinarily, glassware can be satisfactorily cleaned by merely washing well with hot water and soap, using test tube and flask brushes where necessary, rinsing with clear water and draining. Some like

to have their glassware boiled in a 1 per cent soda solution, followed by thorough rinsing. Where it is desired to employ a cleaning fluid for glassware, 10 per cent potassium bichromate in a 10 per cent solution of sulphuric acid makes a good mixture, especially for old slides, greasy glassware, etc. It is not recommended, however, that this mixture be used regularly in a routine way with all glassware, as it is very corrosive.

Cotton Plugs for Test Tubes, etc. For bacteriological use, test tubes, flasks, bottles, etc., are commonly plugged with cotton. Long fiber, non-absorbent cotton is usually utilized for such purpose. The long fiber cotton is preferable to that of short fiber for the reason that the former "flies" less readily when the plug is removed and particles of same are not as apt to enter the tube or flask and possibly contaminate the contents. Some laboratories use absorbent cotton for plugging test tubes, flasks, etc. Such stoppers readily absorb moisture and consequently are more likely to have moulds grow through them and contaminate the material within the flask or tube. This is less likely to happen when non-absorbent cotton is employed.

It is good practice to cover the cotton plugs of flasks, and in some cases large test tubes and bottles, with gauze. This largely eliminates particles of cotton from flying when the plug is removed and, further, aids in maintaining the shape and fit of the plug. It is also a desirable practice to cover the plugged openings of flasks, bottles, etc. containing media or other sterile material with tin foil, or paper, muslin, or gauze, tied in place, before sterilizing. This serves as an added safe-guard against moulds entering the plugs and, further, insures against bacteria collecting on the lip of the flask around the plug with the chances of getting into the container when the plug is removed. Further, the contents of a flask or bottle, thus capped, can usually be poured with less likelihood of contamination.

Test tubes, flasks, pipettes, Petri dishes, in fact most laboratory glassware, are sterilized in the hot air sterilizer at a temperature of 160°C. for one hour.

Pipettes. In preparing pipettes for sterilization, they can be wrapped separately in paper, sterilized, and stored in such condition, or better still, after wrapping they can be placed in metal pipette boxes, sterilized, and removed as required from the container. While the practice is sometimes followed of placing unwrapped pipettes in pipette boxes, sterilizing them, and using from the box as required, it is not a very safe procedure. Small capillary pipettes can be sterilized individually in test tubes or several can be placed in a single test tube.

In addition to a supply of sterile capillary pipettes, the writer has found

it very convenient to have on hand in the laboratory a supply of pieces of glass tubing of about 5 mm. diameter and 6 inches long, plugged with cotton at both ends. Several of these are placed in large diameter test tubes or a large number can be placed in metal sterilizing containers such as are used for pipettes, and sterilized. When it is desired to have a capillary pipette of a particular length or caliber one of these tubes can be drawn out in a flame, making two pipettes all ready for use.

The mouth end of all pipettes used in bacteriological work should be plugged with a small amount of cotton. This plug serves to protect the worker from drawing infectious material into the mouth. The cotton should be placed sufficiently far down in the stem to prevent its becoming wet with saliva. The plug should not be packed in so tightly as to make the drawing up and release of fluid slow and difficult, but, on the other hand, it should be tight enough to insure that it will not be sucked into the mouth or blown into the column of fluid when expelling the contents of the pipette.

Petri Dishes. Petri dishes to be prepared for sterilization can be wrapped separately in paper or several can be wrapped together. Another convenient method is to sterilize a number of them in metal containers made for the purpose.

Ampoules, Phials, etc. Where ampoules, homeopathic phials and small bottles are used in considerable numbers, they can be placed in rows in metal boxes with the open end down, the rows being separated by strips of cardboard, or still better, strips of sheet metal, and sterilized in the hot air oven. Boxes, used in connection with the sterilization of ampoules, and other glassware should have well overlapping lids to insure against bacteria subsequently entering same.

Syringes and Needles. A satisfactory method for maintaining a supply of sterile hypodermic syringes on hand in the laboratory is to wrap the individual syringes in paper, noting the capacity on the outside of the wrapper, and sterilize them in the hot air sterilizer. Before wrapping the syringes the plungers should be removed and the two pieces then wrapped together. Only all glass syringes (Luer type) should be sterilized in the hot air sterilizer. Needles can be placed in small test tubes, one needle to a tube, and then sterilized in the hot air oven.

Swabs. Sterile swabs, so handy around the laboratory, are made by swirling a small bit of cotton around the end of a wooden applicator, placing each one in a test tube, and sterilizing in the hot air sterilizer. If a long applicator is desired the cotton plug for the test tube can be moulded around the applicator leaving part of the stick projecting out of the tube.

Rubber Stoppers and Corks. Rubber stoppers are ordinarily sterilized in

the autoclave. Where only a few rubber stoppers are to be sterilized they can be boiled for one-half to one hour preferably in water to which a small amount of phenol or liquor cresolis compound has been added. Corks can be sterilized either in the hot air sterilizer or autoclave.

Preparation of Slides and Cover Glasses. New slides and cover glasses should be washed well with soap and water, rinsed, then placed in 95 per cent alcohol from which they are removed and thoroughly rubbed between the folds of a clean, soft, lint-free cloth. Previously used slides and cover slips are treated in the same way after having been first boiled and put through cleaning fluid. Just prior to using a slide or cover-glass it is well to pass the same through the flame once or twice to remove any trace of grease which it might contain.

CHAPTER V

Preparation of Culture Media

The preparation of various culture media for the artificial cultivation of bacteria is an important procedure of the bacteriological laboratory. A considerable number of the different species of bacteria, ordinarily dealt with by the average laboratory, can be cultivated on one or more of a few types of media commonly used by practically all laboratories. Other species, however, will grow but scantily or not at all on the more common media, each requiring media adapted to its particular needs. Thus the laboratory keeps on hand what is generally referred to as "ordinary culture media" and, in addition, varieties of the more special media best suited for the cultivation of any exacting organisms which the laboratory might be concerned with.

Aside from supplying nutrition for the growth of bacteria, culture media are of basic importance in the identification of organisms. The behavior of different species in or upon various kinds of media, i.e., the nature of the growth, changes wrought in the media, biproducts, etc., goes a long way towards identification.

Reaction of Culture Media. The reaction of culture media is of great importance as bacteria are very sensitive to variations in the acidity or alkalinity of the media in which they are inoculated. Appreciable departure from the optimum reaction of a medium for the growth of a particular organism will frequently retard or entirely inhibit its growth. Thus, in the preparation of culture media their reaction must be carefully determined and the same properly adjusted to meet the requirements of the organism or organisms to be cultivated in or upon same.

Until recently the phenophthalein titration method was quite generally employed for determining and adjusting the reaction of culture media. This method has now been largely replaced by the more accurate method based on the hydrogen ion concentration of the medium. Such determination can be made electrometrically or colorimetrically. While the electrometric method is the more accurate, the colorimetric method, properly carried out, is ordinarily entirely satisfactory for adjusting the reaction of culture media. Further, the electrometric method requires expensive apparatus and is a much more complicated procedure than the colorimetric method.

Both the old method and the hydrogen ion method of determining and adjusting the reaction of culture media will be given. However, it is strongly recommended that the hydrogen ion determination method be adopted by all laboratories as the standard method.

Method of Determining the Hydrogen Ion Concentration of Culture Media.[1] Before giving the details of the method for the determination of the hydrogen ion concentration of culture media, it will probably be well to give a very brief general discussion of the subject.

Most chemical substances when placed in solution, especially aqueous solution, partially break up or dissociate into ions. Where there is a preponderance of hydrogen ions over hydroxyl ions in such solutions the reaction is acid. When the hydroxyl ions are present in greater concentration than the hydrogen ions the solution is alkaline. When the hydrogen and hydroxyl ions are in balance the solution is neutral.

While the increase of hydrogen ions causes a decrease in hydroxyl ions, and vice versa, neither type of ion is entirely eliminated in aqueous solutions. As the hydrogen ions are increased acidity is approached or increased (if already acid) and the hydroxyl ions are proportionately diminished. With an increase in hydroxyl ions alkalinity is approached or increased (if already alkaline) and the hydrogen ions proportionately diminished. Thus, proceeding from a figure representing neutrality the concentration of one type of ion indirectly indicates the concentration of the other type. Therefore, in place of stating the hydrogen ion concentration of acid solutions and the hydroxyl ion content of alkaline fluids, we merely indicate the hydrogen ion concentration for both acid and alkaline solutions.

The hydrogen ion concentration of a solution indicates the weight, in grams, of its ionic hydrogen per liter of solution. Distilled water, a neutral fluid, contains 0.000,000,1 gram of ionic hydrogen per liter. This figure is taken as a standard of neutrality. While we could indicate the hydrogen ion concentration of water by using the figure mentioned, the same is very cumbersome. This feature can be overcome by using negative exponents. Thus, the hydrogen ion concentration of 0.000,000,1 can be expressed 1×10^{-7} or simply 10^{-7}. While this latter method is better than using the actual weight figure, it is a still better procedure to follow the suggestion of Sorensen and use the symbol "pH" as the logarithm of the reciprocal of the hydrogen ion concentration. Thus, water has a pH of 7.0, such figure representing neutrality in the pH scale.

[1] For a detailed discussion of hydrogen ion determination the reader is referred to The Determination of Hydrogen Ions, by William Mansfield Clark; published by The Williams & Wilkins Co., Baltimore, Md.

Proceeding from this neutral point acid materials scale downward from pH 7 while alkaline solutions increase upwards. In other words, the more acid a fluid the less is its pH, the more alkaline the greater its pH. This is obvious, of course, when it is borne in mind that the greater the hydrogen ion concentration the less the hydroxyl ion content, and the less the figure (negative exponent used logarithmically). Conversely, the greater the hydroxyl ion concentration, the less the hydrogen ion concentration, and the greater the figure.

Sorensen, who first utilized the symbol "pH" did not indicate just why he chose such symbol. The reasons are not material. It is merely a symbol and it need only be borne in mind that "pH" signifies the logarithm of the reciprocal of the hydrogen ion concentration.

While the ionic hydrogen and hydroxyl present in a solution represent but a portion of its total hydrogen and hydroxyl, it is the former type that is of importance in the propagation of microörganisms. Thus, in determining and adjusting the reaction of culture media, the big advantage of the hydrogen ion method over the older titration method lies in the fact that the former determines the concentration of the acidity or alkalinity factors (dissociated ions) which have a direct bearing on the growth of bacteria.

With the old titration method the reaction of a representative specimen of medium is determined and the proportionate amount of normal HCl or NaOH necessary to adjust the entire lot is arrived at mathematically. Because of the presence of variable amounts of buffer material in culture media the hydrogen ion concentration is not increased or decreased in direct proportion to the amount of acid or alkali added. Thus, with the hydrogen ion method the reaction of a batch of medium can not be determined and the amount of acid or alkali to be added to bring it to a certain desired reaction, accurately reckoned mathematically from such initial determination. To clarify this let it be assumed that by adding 25 cc. of N/1 NaOH to 1 liter of bouillon we produce a pH value of 7.4 and when 35 cc. are added a pH of 8.4. If the increase in hydroxyl ions scaled up in direct proportion to the amount of alkali added, then by adding 30 cc. of the N/1 NaOH the pH value should be 7.9. Such is not the case. Hence, in the determination and adjustment of the reaction of culture media by the hydrogen ion method the amount of alkali or acid required to bring a definite amount of the medium to the desired pH value is determined by electrometric or colorimetric methods and the amount of alkali or acid required to adjust the entire batch of medium computed.

Buffers. The term "buffer" is given to such substances, present in solution, as will tend to inhibit a change in pH of the solution when acid or

alkali is added. Peptone, tissue extracts, phosphates, etc., possess buffer qualities.

Colorimetric Indicators. Indicators, used in the colorimetric method of determining hydrogen ion concentration, are substances which assume definite colors and shades of same in solutions of particular hydrogen ion concentrations. Several different indicators are required to cover the entire range of reactions. Those commonly employed were proposed by Clark and Lubs and are given in Table 4.

For bacteriological purposes brom cresol purple, brom thymol blue and phenol red cover the reaction range of the commonly employed culture media. These indicators are ordinarily used in 0.04 per cent alcoholic solution (95 per cent alcohol) in the case of brom cresol purple and brom thymol blue and in 0.02 per cent alcoholic solution in the case of phenol red.

TABLE 4

INDICATOR	COLOR CHANGE	pH RANGE
Thymol blue	Red—yellow	1.2–2.8
Brom phenol blue	Yellow—blue	3.0–4.6
Methyl red	Red—yellow	4.4–6.0
Brom cresol purple	Yellow—purple	5.2–6.8
Brom thymol blue	Yellow—blue	6.0–7.6
Phenol red	Yellow—red	6.8–8.4
Cresol red	Yellow—red	7.2–8.8
Thymol blue	Yellow—blue	8.0–9.6
Cresol phthalein	Colorless—red	8.2–9.8

Color Standards. Sets of comparison color standards, covering the above range of pH 1.2 to 9.8 can be prepared from standard buffer solutions and the various indicators. Unless a laboratory is properly equipped to prepare and test such standards it is best to purchase same. A set of standards covering the range of any one or all of the different indicators can be purchased from the various laboratory supply houses.

Procedure in Adjusting the Reaction of Culture Media. Several 10 cc. pipettes, a burette, test tubes and distilled water are required. It is highly important that all glassware be clean and free from acid and alkali. It is also necessary, of course, to know that the water has been properly distilled. Pipette into each of two test tubes 8 cc. of distilled water and with another pipette 2 cc. of the medium to be adjusted. If the medium contains agar the distilled water should be warm (about 45°C.), to prevent the agar from solidifying. Now add to one of the two tubes 0.50 cc. (or the same amount

contained in color standard tubes) of indicator solution. If, as is usually the case, we are interested only in the final reaction we select the indicator in whose range our desired pH value comes. For example, let it be assumed we are preparing beef infusion broth and we want to adjust the reaction to pH 7.8. We would then use the phenol red indicator. The reaction of the freshly prepared broth would be decidedly acid hence the indicator would

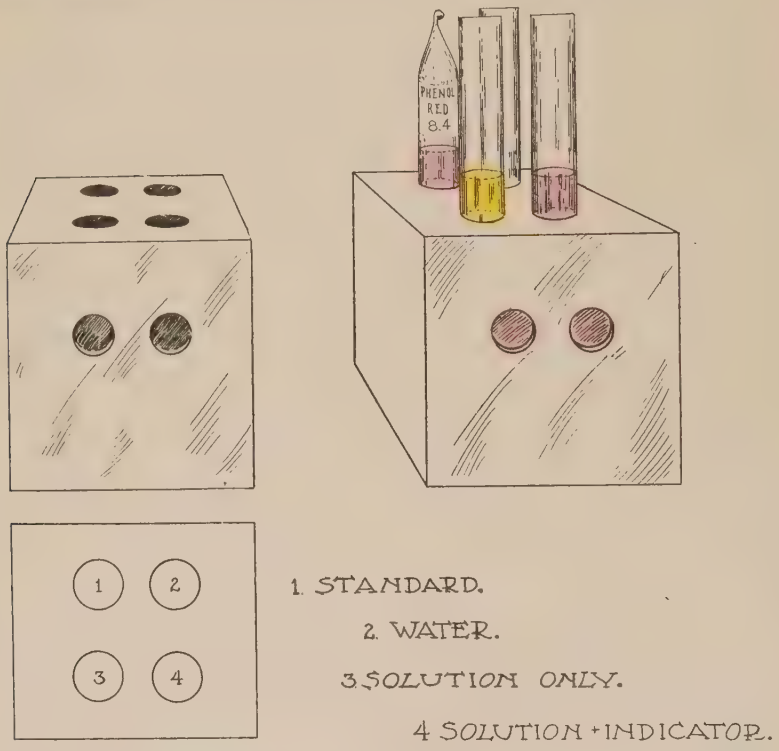


Fig. 10. Comparator Block for Use in the Colorimetric Method of Determining Hydrogen Ion Concentration

give the fluid a more yellowish tinge. Now place in the right front hole of a 4-hole comparator block the tube containing the 8 cc. of water, 2 cc. of medium and indicator. In the hole back of this place a tube containing 10 cc. of distilled water. In the left front hole, place the tube containing the medium solution without indicator and in the hole back of same place the color standard tube of proper pH (7.8). Now from the burette add, drop by drop, N/20 NaOH solution to the tube containing the medium and indi-

cator, shaking the tube as the alkali is added and frequently check its color with the standard as it approaches same. This is done by replacing the tube in the block and holding the same up to the light and comparing colors. When the colors match, the amount of the N/20 NaOH solution required to bring the 2 cc. of medium to pH 7.8 is determined. To adjust the entire lot of medium to such reaction there would, obviously, be required for each liter 500 times the amount of NaOH solution found necessary to adjust the 2 cc. of medium. However, in place of using N/20 NaOH for adjusting the reaction of the bulk of medium we employ a normal solution. Therefore, as the normal solution contains 20 times more alkali, for each liter of the medium we add normal NaOH solution to the extent of 25 times the quantity of N/20 NaOH solution required to adjust the 2 cc. of medium.

Phenolphthalein Titration Method of Adjusting the Reaction of Culture Media. In this method of determining and adjusting the reaction of culture media the following reagents and supplies are required:

10 cc pipettes (graduated)
1 cc. pipettes (graduated)
25 or 50 cc. burette (graduated)
Porcelain casserole
Phenolphthalein solution (0.50 per cent phenolphthalein in 50 per cent alcohol)
N/20 NaOH solution
N/20 HCl solution
Normal NaOH solution
Normal HCl solution

In the preparation of meat base media the initial reaction is usually acid, hence, the alkali solution is most often employed. We will describe the titration of a lot of beef broth medium. The same procedure is followed in titrating a medium of initial alkaline reaction except that the HCl solutions would be employed in place of the NaOH reagents.

Make sure that the various articles of glassware, etc., are scrupulously clean and free from acid and alkali.

Place in the porcelain casserole 45 cc. of distilled water (where agar is to be titrated this should be hot). Add exactly 5 cc. of the medium. Place the casserole over a flame and boil the contents for 2 minutes. Add 1 cc. of the phenolphthalein solution and then from the burette, filled with N/20 NaOH solution, add, drop by drop, sufficient of the alkali to give the solution of medium a faint pink tinge. As the NaOH solution is added the medium should be stirred with a glass rod. Now determine from the burette the amount of N/20 NaOH which was required to neutralize the 5 cc. of medium. From this figure the amount of normal NaOH required to neu-

tralize each liter of medium can be calculated. For example, we find that it required 3 cc. of N/20 NaOH to neutralize 5 cc. of broth. That indicates that 200 times such amount would be required for 1 liter. However, as we employ normal NaOH for neutralizing the bulk of the medium, and as that contains 20 times more alkali than the N/20 solution, to neutralize one liter of medium we add normal NaOH to the extent of 10 times the amount of N/20 NaOH required to neutralize 5 cc. of medium. For example:

5 cc. requires 3 cc. of N/20 NaOH
100 cc. requires 60 cc. of N/20 NaOH or 3 cc. of N/1 NaOH
1000 cc. requires 600 cc. of N/20 NaOH or 30 cc. of N/1 NaOH

As it required 3 cc. of N/20 NaOH to neutralize the 5 cc. of medium we say that the reaction is "+3," that is to indicate that 3 per cent of normal NaOH is necessary to adjust it to the neutral point with phenolphthalein as an indicator. If it is desired to adjust the reaction to +0.50 then we would add only 25 cc. of normal NaOH to every liter of bouillon.

Alkaline reactions are figured in precisely the same way except that the minus sign is used to indicate alkalinity. Thus, to produce a medium of a −1 reaction 1 per cent of normal NaOH is added in addition to the amount necessary to bring it to the neutral point.

PREPARATION OF VARIOUS MEDIA

Beef broth serves as a common base for the preparation of a large variety of culture media. Such broth may be prepared from fresh beef or it may be made from concentrated beef extract such as Liebig's or Armour's. The beef infusion broth, prepared from fresh beef, is preferable to the beef extract broth. However, the latter is very simple of preparation and will suffice for the growth of a large number of bacteria.

Beef Infusion Broth. Fresh beef, freed from as much fat and fascia as possible, is ground in a sausage mill or food-chopper. To every 500 grams of the ground beef add 1 liter of distilled water (or tap water) and allow the mixture to stand in the refrigerator over night. The next morning heat the mixture to 60°C. and maintain at such temperature for one hour. Filter the fluid through cheese cloth. The meat residue is then put into a fruit press, or if such is not available, a heavy cloth which will withstand twisting, and as much more fluid as possible extracted from the tissue. Determine loss of fluid and make up to original volume by adding hot water. (If desired this can be filtered and then sterilized in flasks or bottles and stored as "beef infusion" for the future preparation of broth.) For every liter of beef infusion add 10 grams of peptone and 5 grams of sodium chlo-

ride. Heat the mixture to boiling. Titrate, adjust the reaction and filter first through cotton and then through filter paper. Fill into flasks or tubes and sterilize in autoclave at 15 pounds pressure for one-half hour.

Where large quantities of media are used and storage space is a factor, in place of the usual beef infusion it is frequently found convenient to keep on hand a double-strength beef infusion as stock for broth. In the preparation of this only 1 part of water is added to 1 part of beef, the additional volume of water being supplied when the standard broth is made.

Beef Extract Broth. To each liter of water add 5 grams of Liebig's or Armour's beef extract, 10 grams of peptone and 5 grams of sodium chloride. Heat to boiling and maintain at same for about 10 minutes. Determine reaction and adjust to that desired. Filter through cotton and then through filter paper. Place in flasks or tubes and autoclave for one-half hour at 15 pounds pressure.

It should be remembered that when broth, especially beef-infusion broth, is autoclaved such treatment results in a change in the reaction (increased acidity) which may be sufficient to cause trouble when utilized for the growth of exacting bacteria. Due allowance should be made for such change when adjusting reactions by making acid media a little less acid than is desired in the final product, and alkaline media a little more alkaline, before sterilization. A specimen of the final sterilized product should be titrated to make sure the reaction is satisfactory.

Glycerin Broth. Glycerin broth is prepared by adding from 0.5 to 7 per cent glycerin to ordinary broth, the amount of glycerin added varying for different purposes. For the growth of Mycobacterium tuberculosis, broth containing 6 to 7 per cent glycerin is employed.

Sugar-Free Broth. Inoculate the desired quantity of beef infusion or extract broth with a generous amount of a 24-hour culture of Escherichia coli and incubate from 24 to 48 hours, depending upon the amount of medium. Place in the Arnold sterilizer for one-half hour to destroy the organisms. Titrate and adjust reaction. Before filtering it is usually necessary to employ special means for clearing the medium. This is accomplished in the following manner:

After medium has cooled down to 50°C. or less add the white of one egg beaten up in about 25 cc. of water, for every liter of the bouillon, stirring well. Bring up to boiling. Filter through paper until clear. Fill into flasks and sterilize in autoclave at 15 pounds pressure for one-half hour.

Passing the medium through the same filter paper several times frequently clears up a cloudy medium. Filtration through a pad of asbestos wool in a Buchner funnel or through a coarse Berkefeld filter, while not ordinarily necessary, will clear such medium perfectly.

Carbohydrate Media. Various fermentable substances added to culture media are of value in identifying certain bacteria. The agents most commonly used are given below. They are added to slightly alkaline, sugar-free broth, usually in 1 per cent amounts, filled in fermentation tubes and sterilized in the Arnold sterilizer for one-half hour on three successive days. Some of the sugars are extremely susceptible to heat, being split or converted at high temperatures. It is highly important that precaution be taken to prevent this. The autoclave must not be used for the sterilization of media containing such agents. Fractional sterilization in the Arnold sterilizer usually gives satisfactory results but even this method is not absolutely reliable. The very best procedure is to sterilize fermentable media by Berkefeld filtration.

Where sterilization is to be accomplished by filtration through Berkefeld filters the following procedure should be followed: Sterilize a definite amount of sugar-free broth in the autoclave and allow it to cool down to about 50°C. If broth is on hand, already sterilized, heat to approximately 50°C. Add 2 per cent of the desired fermentable substance to the warm broth and shake until well dissolved. The larger amount of sugar or other fermentable agent is added because some is lost in filtration. The broth is then filtered through a normal ("N") Berkefeld filter into a sterile flask or bottle. It is then filled into fermentation tubes in the same manner as we would proceed to fill bacterins or sera into ampoules or vials, taking precautions to prevent contamination.

It is common practice to put a small amount of indicator, such as phenol red, into sugar media to indicate the production of acid. A few drops of a 1 per cent solution of phenol red to each 100 cc. of medium gives the slightly alkaline broth a reddish tinge. With the production of acid the broth loses its red color and takes on a yellowish tinge.

The following are some of the agents employed for fermentation reactions: Dextrose, saccharose, lactose, maltose, levulose, galactose, raffinose, dextrin, inulin, rhamnose, xylose, arabinose, mannite, dulcite, glycerin, and salicin.

Martin's Peptone Broth. Several pigs' stomachs are obtained, washed and the fat and any attached tissue removed. They are then finely ground by passing them through a food-chopper. The tissue is then placed in glass flasks or bottles and to every 300 grams 1500 cc. of distilled water, heated to 50°C., and containing 15 grams of HCl (c.p.) are added. The mixture is kept at a temperature of approximately 50°C. for 24 hours. After the 24 hours digestion the material is heated to 80°C. to stop the process. It is then filtered through cotton and if the temperature of the filtrate is below 70°C. it is raised to such point and neutralized to litmus. Sterilize in autoclave for one-half hour at 15 pounds pressure.

Dunham's Peptone Solution. Peptone solution is commonly employed to determine whether or not an organism produces indol. It is merely a solution of peptone and sodium chloride in water. To 1 liter of distilled water add 10 grams of peptone and 5 grams of sodium chloride. Heat to effect solution, filter through filter paper and tube. Sterilize in the autoclave at 15 pounds pressure for one-half hour.

Tests for Nitrites and Indol. Indol is produced as a result of the action of certain bacteria on the tryptophane content of the peptone employed. As the tryptophane content of various grades of peptone has been found to vary considerably it is essential that a good grade of such product, made especially for bacteriological work, be employed.

For the determination of indol production, cultures grown for several days should be used, although with some organisms the reaction can frequently be demonstrated after only 18 to 24 hours incubation.

Dunham's peptone solution is commonly employed because it is a sugar-free medium. In utilizing other media for indol tests they must be sugar-free, as acid, formed by the splitting of carbohydrates, interferes with indol production.

The common method of testing for indol consists of the addition of 8 or 10 drops of concentrated sulphuric acid to the culture. If, in addition to indol, nitrites are present as a result of the bacteria reducing nitrates, a positive reaction results without the addition of any other reagent. However, if the *pink color*, which is indicative of the presence of indol, does not appear following the addition of the acid a small amount of a very dilute solution of sodium nitrite (1 cc. of a freshly prepared 1:10,000 solution) is added. If the pink color does not appear within a few minutes the test is considered negative.

Vanillin Test for Indol. This is a very satisfactory and frequently employed test for indol production. In carrying out the test 5 drops of a 5 per cent alcoholic solution of vanillin are added to 5 cc. of the culture. Following this 2 cc. of concentrated sulphuric acid are added. In the presence of indol an orange color develops within 2 or 3 minutes. Tryptophane will also give a color reaction to this test but it is readily distinguished because it is reddish violet and appears more slowly, becoming deeper in hue on standing and when the solution is heated.

Ehrlich's Method for Detecting Indol. Ehrlich's method for demonstrating the presence of indol is probably the most reliable of all such tests. The test is conducted by adding 1 cc. of a 2 per cent alcoholic solution of paradimethylaminobenzaldehyde to the culture and then, drop by drop, concentrated hydrochloric acid until a red zone appears at the junction of the

peptone solution and the alcohol. The amount of hydrochloric acid required is not more than 0.5 cc. On standing the color zone becomes deeper and wider. The color material is chloroform soluble. Thus, in order to confirm the test, chloroform should be added and the tube shaken to demonstrate solubility of the red coloring matter.

Medium for Voges-Proskauer Reaction. To 500 cc. of Dunham's peptone solution (prepared as outlined above) add 10 grams of dextrose. Tube and sterilize.

The method of making the test is as follows: Inoculate a tube of the medium with the organism in question and incubate for 72 hours. Add 2 cc. of a 50 per cent potassium hydroxide solution. Observe during the following 24 hours. Frequent shaking of the tube hastens a positive reaction by promoting oxidation. A positive reaction is denoted by a pink color resulting from the presence of acetyl-methyl-carbinol.

Litmus Milk. Place 1 liter of fresh, unpasteurized milk in an Erlenmeyer flask and heat in the Arnold sterilizer for one-half hour. Place in the ice-box over night (several hours will do) to allow the butter fat to rise. Carefully siphon off the milk below the cream line. Add sufficient litmus to give the product a distinctly bluish tinge, tube and sterilize in the Arnold for one-half hour on each of 3 successive days.

Milk Whey Medium. Allow the desired quantity of fresh, unpasteurized milk to stand in the ice-box a few hours until the cream separates. Carefully siphon off the milk, discarding the cream. Heat the milk to 95°C., stirring continually to prevent the formation of a "skin." Add 2 cc. of glacial acetic acid for every liter of milk to coagulate the casein. Filter through cheese-cloth. Add 1.50 per cent peptone and 0.5 per cent sodium chloride and heat to boiling. Titrate and adjust the reaction to pH 6.8. Filter through cotton and then through filter paper. Sterilize in autoclave at 15 pounds pressure for one-half hour. If it is desired to have the medium particularly clear it is frequently necessary to use egg white for clearing.

Liver Broth. This is prepared in the same way as beef infusion broth, substituting beef liver for the muscle tissue.

Veal Broth; Pork Broth. These are prepared according to the method outlined for beef infusion broth, substituting the desired meat for the beef tissue.

Potato Broth. Wash and pare a number of sound white potatoes and mince them with a food-chopper. Add 2 parts of distilled water to one part of chopped potatoes and allow same to stand 16 to 18 hours in the ice-box. Heat to boiling, then filter the mixture through cheese-cloth and allow the filtrate to cool to about 45°C. Add egg white mixed in a little water

(white of 1 egg for each liter of broth) and bring to boil. Filter through cotton. Fill into flasks and sterilize in the autoclave at 15 pounds pressure for one-half hour.

Lactose-Bile Medium. To 200 cc. of fresh ox bile add 2 grams of peptone and 2 grams of lactose. Fill into fermentation tubes and sterilize for one-half hour on three successive days in the Arnold sterilizer. If fresh bile is

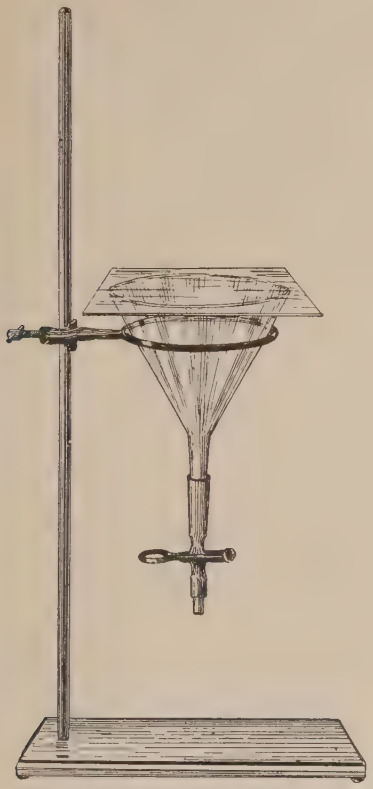


FIG. 11. APPARATUS FOR TUBING MEDIA

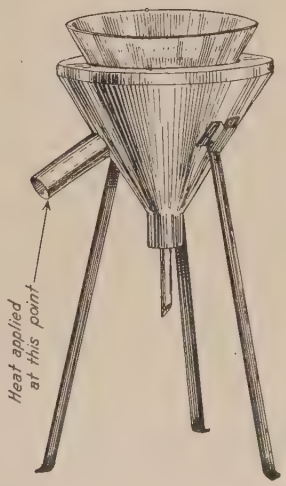


FIG. 12. HOT WATER FUNNEL

not available a 15 per cent solution of commercial, inspissated ox bile may be used.

Hiss Serum-Water Medium. A serum-water medium is sometimes used in place of sugar-free broth as a vehicle for the various sugars employed in fermentation tests. Add 3 parts of distilled water to 1 part of beef serum. Heat for 15 minutes in the Arnold sterilizer, remove and add sufficient

litmus to give the fluid a bluish color. Then add 1 per cent of the sugar desired and sterilize fractionally in the Arnold.

Serum Broth. In the preparation of this medium 10 per cent of sterile beef, horse, or sheep serum is added to sterile beef infusion broth and the same tubed with precautions to prevent contamination.

If it is not desired to keep a stock of this medium on hand it can be prepared as required by adding approximately 10 per cent serum to tubes of bouillon as they are used.

Dextrose-Serum Broth. This is prepared in the same manner as serum broth except that 1 per cent dextrose broth is used in place of the plain bouillon.

Blood Broth. This is prepared by adding 10 per cent of sterile, defibrinated blood to beef infusion broth. Horse, sheep or rabbit blood is usually employed. It should be taken with aseptic precautions from the jugular vein of a horse or sheep or by cardiac puncture from a rabbit. If taken in the laboratory from a rabbit and used immediately it need not necessarily be defibrinated.

Nutrient Agar. To one liter of beef infusion or extract bouillon add 17.50 grams of powdered, granular or shredded agar. Where shredded agar is used moisten the weighed amount with a little water and put it through a food-chopper before adding it to the bouillon.

When powdered or granular agar is employed the same will form clumps if added directly to the bouillon. This can be obviated by putting the weighed quantity of agar in a mortar and making a paste of same with a little of the bouillon and finally adding the paste to the bulk of the broth.

The agar can be best dissolved by adding it to the bouillon and placing the container in the autoclave at 15 pounds pressure for one-half hour. If heated over an open flame it is best to use a double-boiler with a 20 per cent sodium chloride solution in the outer container rather than plain water. After heating over an open flame the loss by evaporation should be made up.

After the agar has been thoroughly melted it should be titrated, the reaction adjusted, and then filtered while hot through a cotton filter, the cotton having been previously moistened with a little hot water. If desired a hot-water funnel can be used to hold the glass funnel carrying the cotton filter. This tends to keep the medium hot thereby lessening the danger of the product solidifying if filtration is slow. After filtering, the agar is placed in tubes or flasks and sterilized at 15 pounds pressure for one-half hour. Following sterilization tubes containing medium intended for stab cultures are allowed to stand in an upright position. For slant cultures the tubes are placed in a

flat position with the plugged ends elevated just sufficiently to give the medium the desired slant.

Glycerin Agar. This is merely nutrient agar plus 3 per cent glycerin.

Glucose Agar. Glucose agar is made by adding 2 per cent agar to glucose broth containing 1 per cent glucose. It should be sterilized by the fractional method.

Maltose Agar (for Fungi) (Sabouraud). To 500 cc. of distilled water add 7.50 grams of agar and 5 grams of peptone. Heat until thoroughly dissolved. Measure and bring volume up to 500 cc. Add 20 grams of maltose and heat again slightly, if necessary, to dissolve this. Place in tubes or small flasks and sterilize fractionally.

Serum Agar. Melt the contents of a flask of sterile nutrient agar (2 per cent agar is best) and allow same to cool down to 58°C. Add, with aseptic precautions, 10 per cent of sterile horse, beef, sheep or rabbit serum, which has been heated to 58°C. Tube immediately taking precautions to prevent contamination.

If large quantities of serum agar are to be tubed, it is necessary to have an arrangement whereby the temperature can be maintained sufficientiy high to prevent solidification during the tubing process. This can be accomplished by having the melted agar in an Erlenmeyer flask fitted with rubber stopper and siphon. The flask can be kept in a water bath maintained at 55° to 58°C.

Blood Agar. Add 10 per cent of sterile defibrinated horse, sheep or rabbit blood to a definite amount of sterile nutrient agar (preferably 2 per cent agar) which has been completely melted and allowed to cool down to 45°C. The tubes or flasks containing the melted agar should be placed in a water bath maintained at 45°C. and be removed as the blood is to be added to them. Care must be exercised to obtain the blood in a sterile condition and aseptic precautions must be taken in adding it to the melted sgar.

Nutrient Gelatin. Heat 1 liter of beef extract or beef infusion broth to 60°C. and dissolve in it 175 grams of Hesterberg's "Gold Label" or other good grade of gelatin. Titrate and adjust the reaction to pH 7.6. Add the whites of two eggs (to clear) and bring the temperature of the mixture up to the boiling point. Filter, preferably through paper, tube and sterilize in Arnold sterilizer for one-half hour on each of three successive days.

Chocolate Agar. Some organisms grow best on media containing blood which has been changed from a deep red to a chocolate color by heating the medium at a high temperature. This medium is prepared in the following manner: Melt the desired amount of nutrient agar of proper reaction. Allow it to cool to 55°C. and then add 10 per cent of defibrinated horse,

sheep or rabbit blood. Gradually raise the temperature to 85°C. The mixture assumes a chocolate color. It is then immediately tubed or poured into Petri dishes under aseptic conditions.

Eosin-Methylene-Blue Medium (Teague's Medium). This medium is of great value in work with organisms of the colon-typhoid group.

Prepare 1 liter of nutrient agar using either beef extract broth or sugar-free beef infusion broth. Adjust the reaction to pH 8.0 and add 0.5 per cent lactose and 0.5 per cent saccharose. Sterilize by the fractional method. Following the last sterilization add 20 cc. of a 2 per cent yellowish eosin solution and 20 cc. of a 0.5 per cent methylene blue solution. Mix the dye well through medium by shaking it and then pour into Petri dishes.

Colon colonies appear black with a metallic lustre whereas typhoid and paratyphoid organisms appear transparent and colorless.

Lactose-Litmus Agar. This is another valuable medium for use in identifying organisms of the colon-typhoid group.

To 1 liter of melted agar, prepared from beef extract or sugar-free beef infusion broth, add sufficient litmus to give the medium a distinct lilac color. The reaction should be about pH 8.0. While hot add 1 per cent lactose and sterilize fractionally in the Arnold sterilizer. Immediately following the third sterilization pour the medium into Petri dishes.

Colon colonies are red with a zone of similar color around the colony. Typhoid and paratyphoid colonies are colorless.

Endo's Medium. Endo's medium has been of great value in dealing with bacteria of the colon typhoid group. On this medium colon organisms produce red colonies while typhoid and paratyphoid colonies are colorless.

A neutral beef extract broth containing 3 per cent agar is used as a base for Endo's medium. This can be prepared, sterilized and stored in flasks of convenient size for use. It is advisable not to make Endo's medium in larger quantities than will be used in a short space of time. When ready to prepare a lot of such medium melt the contents of one or more flasks. For each 100 cc. of medium prepare the following: Dissolve 1 gram of lactose in 15 cc. of hot distilled water. In another test tube or flask dissolve 0.25 gram of anhydrous sodium sulphite in 10 cc. of water and to this add 0.50 cc. of a saturated solution of basic fuchsin in 95 per cent alcohol. Now add the fuchsin-sulphite mixture to the lactose solution and pour the whole into the flask of melted agar. Mix well and pour the medium into Petri dishes and allow it to harden.

Endo's medium should be colorless or have a very faint salmon color. Colonies of colon organisms appear pink or red on this medium while colonies of the intermediate group and the typhoid organism appear colorless.

Petroff's Medium. This is a very valuable medium for the direct isolation of M. tuberculosis. Place 500 grams of ground beef in 500 cc. of water containing 15 per cent glycerin. Keep the mixture in the refrigerator 24 hours and then extract the fluid by putting the material through a press. Filter through cotton, and paper and finally through a Berkefeld filter to sterilize. Add 1 cc. of a 1 per cent alcoholic solution of gentian violet to every 100 cc. of filtrate. Next immerse several eggs in 70 per cent alcohol and allow them to remain in same for 10 or 15 minutes to sterilize the shells. Carefully break the eggs into a sterile beaker or mortar and beat them up well with a glass rod or pestle. Filter through sterile gauze into a sterile container. Now add one part of the broth to two parts of egg. Mix thoroughly and fill into sterile tubes. Slant the tubes in inspissator and run same at 85°C. for one hour on each of three consecutive days.

If this medium is to be employed for the isolation of the bovine type of M. tuberculosis proceed as outlined above but omit the glycerin.

Dorset's Egg Medium. Thoroughly wash 8 eggs and then allow them to remain immersed in 70 per cent alcohol for 10 or 15 minutes. Carefully break them into a sterile flask. Break the yolks with a sterile glass rod and then mix the yolks and whites thoroughly by gentle shaking. Avoid the formation of froth and bubbles as much as possible. Add 50 cc. of sterile distilled water and twirl the flask a little more to mix this in. Strain the mixture through several thicknesses of sterile gauze and then fill it into sterile tubes placing about 10 cc. in each tube. Slant the tubes in the inspissator and raise the temperature to 73°C. maintaining it at such point for $4\frac{1}{2}$ hours. Heat at the same temperature over the same period of time on the second day. On the third day heat at 76°C. for 3 hours. Paraffin the plugs or put tight-fitting rubber caps over same. Just before inoculating add 3 or 4 drops of sterile distilled water to each tube.

Glycerin-Egg Medium (*Lubenau's*). Prepare a quantity of veal infusion broth using 0.50 per cent sodium chloride and 2 per cent peptone. Sterilize in autoclave. Add 5 per cent glycerin. Titrate and adjust the reaction to pH 7.0. For every 200 cc. of this broth 10 eggs are required. The eggs are thoroughly washed and then sterilized by placing them in 70 per cent alcohol and allowing them to remain in same for 15 minutes. Break the eggs in a sterile flask and mix the yolks and whites thoroughly as described in the preparation of Dorset's egg medium. Next add the broth gently shaking the flask. When a homogeneous mixture is obtained, tube and inspissate in the manner described for Dorset's medium.

Potato Medium. Carefully select a number of large, sound, white potatoes. Scrub them thoroughly with a stiff brush and then pare them

taking off a rather thick peel. Wash them in running water and then cut as many cylinders as can be gotten out of each potato with an apple corer or large cork borer. Next cut the cylinders into pieces having a square cut base and slanting or wedge-shaped top. Wash in running water over-night. Place each plug, slanting end up, in a sterile test tube containing a pledget of absorbent cotton wet with distilled water. Sterilize in the autoclave at 15 pounds pressure for one-half hour.

Glycerin-Potato Medium. Proceed the same as in the preparation of potato slants. After washing the potato wedges allow them to soak for one hour in a 6 per cent solution of glycerin. Place in tubes containing pledgets of cotton well moistened with the glycerin solution and sterilize them in autoclave at 15 pounds pressure for 30 minutes.

Loeffler's Serum Medium. To 1 part of sterile, neutral, beef infusion broth containing 1 per cent dextrose add 3 parts of sterile beef, horse or sheep serum. Tube with aseptic precautions and place the tubes in a slanting position in the inspissator and slowly raise the temperature to 85°C. Maintain at this temperature for one hour. On each of the two succeeding days sterilize for one hour in the Arnold sterilizer.

Starch Agar (Vedder). Infuse 500 grams of lean ground beef in 1000 cc. of distilled water over night in the refrigerator. Bring to a boil and filter through muslin. Add 1.75 grams of agar and boil until dissolved. Titrate and adjust reaction to pH 6.8. Allow the filtrate to cool down to about 50°C. and then add the white of an egg and again bring to boil. Filter through cotton. Add 1 per cent of corn starch to the filtrate and place it in the Arnold sterilizer for 45 minutes. Shake the flask well several times during the heating process to get the starch evenly distributed. After removing from the Arnold sterilizer tube, and sterilize in the autoclave for 20 minutes at 10 pounds pressure.

While this medium was originally used for the growth of meningococci it has proved of value for the propagation of organisms of the hemorrhagic septicemia group, streptococci, etc.

Hemoglobin Agar. Thirty cubic centimeters of horse, sheep or rabbit blood is drawn with aseptic precautions and immediately placed in a flask containing 200 cc. of sterile distilled water. Twirl the flask rapidly until the blood cells are thoroughly laked. This hemoglobin solution is then added with aseptic precautions to tubes or flasks (for plates) of melted nutrient agar cooled down to about 55°C. Approximately 10 per cent of the hemoglobin solution is added to the agar. The tubes are slanted or the material in the flask is poured into Petri dishes and the medium allowed to harden.

Hemoglobin Broth. Prepare a solution of hemoglobin as described above. While not absolutely necessary, it is preferable to filter the solution through Berkefeld filter to free it from any unlaked cells or suspended cell debris. Add approximately 10 per cent of this solution to tubes of nutrient broth, observing precautions to prevent contamination during the process.

Russell's Double Sugar Medium. As a base for this medium a beef extract broth containing 2.5 per cent agar and having a reaction of about pH 7.6 is employed. A quantity of such agar is melted and 0.25 cc. of a 1 per cent aqueous solution of neutral red added as an indicator. Then add 1 per cent lactose and 0.1 per cent glucose dissolved in a small amount of hot water. Mix thoroughly, place in tubes and sterilize fractionally in the Arnold sterilizer. Following the last sterilization slant the tubes.

This medium is of great value in identifying organisms of the colon-typhoid group. The medium is inoculated by first streaking the surface of the slant with a needle containing a little material from the questionable colony. Finally, before withdrawing the needle, it is stabbed into the butt of the medium.

Es. coli produces a maximum amount of gas (both the lactose and glucose being fermented) as indicated by numerous gas bubbles and breaks in the medium. The red color is lost in both the butt and slant. Organisms of the Salmonella group produce only a small amount of gas (the 0.1 per cent glucose alone being fermented). The butt loses its red color whereas the slant retains it. The typhoid organism produces no gas but acid is formed in the depths of the medium so that with this organism the butt appears yellow, the slant red and there are no gas bubbles.

Dextrose-Serum Agar. Prepare a quantity of nutrient agar using a sugar-free broth and 2.5 per cent agar. Titrate and adjust the reaction to pH 7.2. Add 1 per cent dextrose dissolved in a few cubic centimeters of hot water. Tube and sterilize for one hour on three successive days in the Arnold sterilizer. Immediately following the third sterilization place the tubes in a water bath at 60°C. to prevent solidification and then add to each tube approximately 10 per cent of sterile horse, beef, or sheep serum, observing precautions to prevent contamination.

A vaccine filling burette with the filling point protected by a bell glass is of great value in adding sterile sera, exudates, etc., to culture media (see page 466).

Calcium Carbonate Broth. Certain bacteria growing in artificial culture media, particularly those containing carbohydrates, are frequently retarded in propagation by the production of acid before a heavy growth is obtained. This is notably true of the streptococci. This can be overcome to a certain

extent by adding to dextrose broth 1 per cent calcium carbonate either in powder form or in small lumps (marble). The calcium neutralizes the acid produced thereby permitting a more luxuriant growth of the organism.

The calcium carbonate should be first sterilized in the hot air sterilizer and then added to the broth before the latter is sterilized by the fractional process.

"Hormone" or Vitamine Agar. Cole and Lloyd[2] demonstrated that in the process of filtering culture media through cloth, cotton or paper, certain "vitamins" originally referred to as "hormones" were removed. This has been confirmed by numerous investigators and a number of methods have been described for the preparation of culture media by processes which will conserve these hormones. The method most commonly employed is that recommended by Huntoon[3] or a modification of same.

Cut up one or more fresh beef hearts and put the tissue through a food-chopper. To every 500 grams of minced heart muscle add 500 cc. distilled water, 10 grams of peptone, 5 grams of sodium chloride and a whole egg well beaten. In another kettle place 500 cc. of distilled water for every 500 grams of heart tissue in the first container. Add 3 per cent agar and bring to boil, continuing the heating until the agar is thoroughly dissolved. In the meantime heat the first mixture to 70°C. (the meat turns brown at this temperature). Strain through a wire strainer. After the agar solution has cooled down to about 70°C. add this to the broth. Titrate and adjust reaction to pH 7.6. Place the kettle containing mixture in the Arnold sterilizer for one hour. Do not remove medium immediately from sterilizer but allow it to sediment and harden. After the agar has "set" it can be carefully turned out of the kettle and a layer of the medium containing the bulk of the sediment can be trimmed off and discarded. Replace the remainder in the Arnold and after the medium has melted tube and sterilize by the fractional method. Some laboratories add 1 per cent glucose to their hormone agar.

If a Sharpless centrifuge is available the medium can be cleared by running it in such apparatus rather than by the sedimentation process.

Lead Acetate Agar. Lead acetate agar is of value in differentiating Salmonella paratyphi (Paratyphosus "A") and Salmonella schottmülleri (Paratyphosus "B").

This medium is prepared by adding sufficient of a 0.25 per cent solution of basic lead acetate to melted agar, cooled down to 60°C., to give a 0.05 per

[2] Cole and Lloyd, Jr. Path. and Bact., vol. 21, 1916.

[3] Huntoon, Jr. Inf. Dis., vol. 23, 1918.

cent concentration of the lead acetate. The medium is tubed and permitted to harden with the tubes in the upright position.

The medium is inoculated by "stabbing" it deeply several times with the needle carrying the inoculum. Salmonella paratyphi does not discolor the medium. Salmonella schottmülleri, and other organisms of the "B" group (S. enteritidis, S. typhi-murium) blacken it.

Meat Mash Medium for Anaerobes. To 500 grams of finely ground, lean beef add 500 cc. of distilled water, 1 per cent peptone and 0.5 per cent sodium chloride. Bring the mixture to a boil and skim it. Titrate and adjust the reaction to pH 7.6. Add 1 per cent glucose dissolved in a small amount of hot water. Fill into tubes, shaking the mixture frequently during the process to keep the meat particles evenly distributed. It is well to fill the tubes about half full. Put a half-inch layer of paraffin oil in each tube and sterilize fractionally.

Brain Medium (Hibler). Place 500 grams finely ground beef liver in one container and a like amount of minced beef brains in another container. Add 1 liter of distilled water to the liver. Place both containers in the Arnold sterilizer and heat for one hour in the flowing steam. Remove and strain the liver broth through cheese-cloth and then through cotton. Add 1 per cent peptone and 0.50 per cent sodium chloride and adjust reaction to pH 8.2. Put up in tubes placing in each one part of the cooked brain tissue and two parts of the liver broth. Sterilize in the autoclave at 15 pounds pressure for one hour.

This medium is used for the growth and identification of various anaerobes. Some blacken the brain tissue while others do not.

Nicolle, Novy, MacNeal Medium ("N.N.N." Medium). This medium has been of great value in the cultivation of protozoan organisms such as trypanosomes, leishmania, etc.

To 900 cc. of distilled water add 14 grams of agar and 6 grams of sodium chloride. Boil until the agar is thoroughly dissolved. Tube and sterilize in the autoclave at 15 pounds pressure for one-half hour. Keep this as stock and as a batch of the "N.N.N." medium is required melt the contents of the desired number of tubes and cool the same down to 48°C. Then add $33\frac{1}{3}$ per cent of sterile, defibrinated rabbit's blood. Mix thoroughly and slant the tubes. Paraffin the plugs or use rubber caps to prevent evaporation of the water of condensation.

This medium is inoculated with infected blood or other fluid containing the organisms to be cultivated. Cultures are usually incubated at room or lower temperature.

One-tenth Per Cent Agar (Hitchens). This medium consists of a beef infu-

sion broth to which only 0.10 per cent agar is added. The medium has proved very valuable for the cultivation of anaerobes.

Serum-Glucose-Cystine-Agar (*Francis*). This medium is used primarily for the cultivation of Pasteurella tularense but is valuable for the propagation of streptococci, etc.

Melt 500 cc. of a stock beef infusion agar containing 1 per cent peptone, 0.50 per cent sodium chloride and 1.50 per cent agar and of pH 7.4 reaction. Add 0.50 gram of cystine and 5 grams of glucose. Place in the Arnold sterilizer and subject to flowing steam for 15 minutes. Cool to 50°C., add 25 cc. of sterile horse serum and tube with aseptic precautions. Slant the tubes and after hardening incubate for 24 hours to determine sterility.

CHAPTER VI

Methods Employed for the Artificial Propagation of Bacteria

In the isolation and cultivation of microörganisms a number of important factors must be taken into consideration. First of all, if the known or suspected organism is cultivatible a medium suitable for its growth must be selected. If the material to be cultured is known or believed to contain more than one organism methods must be employed to separate the different bacteria so that each may be isolated in pure culture or the organism we are interested in be separated from contaminating organisms.

The method of inoculation must be given attention. Some organisms will grow readily if the inoculum is merely streaked across the surface of some solid medium or inoculated into a tube of fluid medium. On the other hand, M. tuberculosis, for example, would fail to propagate if inoculated below the surface of broth, in such medium it must be floated on the surface. Then some organisms grow much better when rubbed into the surface of a medium than when lightly streaked across it.

In the incubation of cultures of various bacteria temperature requirements must be taken into consideration. A large percentage of the pathogenic bacteria will grow at the ordinary incubator temperature of 37.5°C. even though such temperature is not their optimum. On the other hand, there are some organisms which are more fastidious in their temperature requirements and will not propagate if incubated at temperatures appreciably removed from their optimum.

Atmospheric conditions are of vast importance in dealing with the two great divisions of bacteria (aerobes and anaerobes). Anaerobes would be entirely missed if we employed only aerobic methods in our culturing. Likewise, strict aerobes would fail to grow under anaerobic conditions. Surface tension, the presence or absence of various gases, or the amounts of same in the atmosphere, are of importance although our knowledge of these factors is not, by any means, complete. The moisture content of the atmosphere has a direct influence on the propagation of bacteria, a very low humidity not being conducive to best results.

Inoculation of Culture Media. Culture media can be inoculated through the use of platinum loops and needles, pipettes, capillary pipettes, glass rods (spreaders), sterile swabs, etc.

Platinum Loops and Needles. Platinum loops are used to streak material over the surface of solid culture media or into fluid media. For the preparation of loops platinum wire of 26 or 27 gauge is most satisfactory. Take a 4-inch piece of such wire and fashion a loop at one end by twisting it around a piece of 4 mm. wire. The wire can then be fixed in a glass handle. This is accomplished as follows: Heat one end of an 8-inch piece of glass rod about 6 mm. in diameter in a Bunsen flame until it softens, at the same time holding the straight end of the platinum wire in the flame with a pair of forceps. Push about $\frac{1}{4}$ to $\frac{3}{8}$ inch of the red hot wire into the molten glass and allow to cool. If desired Kolle needle holders can be employed. However, the glass holders are entirely satisfactory.

In making "stab" inoculations the platinum needle is employed. Usually a wire of a little heavier gauge than that used for the loop is desirable as it bends less readily. It is mounted in a handle in the same way as the loop.

For the inoculation of pieces of pellicle of M. tuberculosis a wire with the loop bent at right angles to the rest of the wire is desirable. A piece of

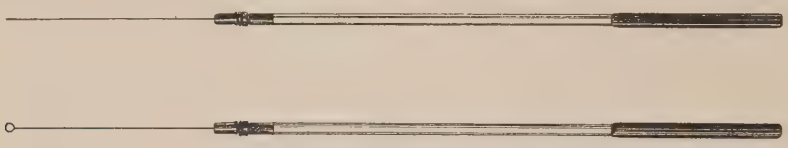


FIG. 13. PLATINUM INOCULATING LOOP AND NEEDLE

pellicle can be readily lifted from the seed culture and floated on the broth to be inoculated by inserting the needle straight down in the fluid.

Platinum loops and needles are sterilized immediately before and after each inoculation by heating to glowing in the flame of a Bunsen burner. While the platinum wire cools very rapidly one should wait a few seconds after heating the wire before picking up the inoculum. After the inoculation is made care must be taken to prevent spluttering of the infectious material left on the loop or needle. This occurs when a considerable amount of moist material remains on the wire or when cultures or material containing waxy or fatty constituents are handled. This can usually be avoided by gradually heating the loop to glowing rather than plunging it into the flame and allowing it to remain until red hot. Another safe method which is desirable when numerous transfers of M. tuberculosis or other highly pathogenic organisms are made, is to plunge the loop first into a bottle of concentrated sulphuric acid and then flame it.

In making an inoculation of bacteria from a culture to a fresh tube of medium proceed as follows: Hold the tube containing the culture and the

tube of fresh medium in the left hand, having the butts of the tubes in the palm of the hand. Pick up the platinum loop in the right hand holding it with the thumb and forefinger in much the same manner as a pen is held. Now heat the platinum wire red hot in a Bunsen flame and then remove it. Next ignite the cotton stoppers of the two tubes by passing them into the flame and after they have burned a second or two extinguish the flame. Now with the right hand, which is still holding the sterile platinum loop, catch one of the cotton plugs between the little and ring fingers and remove it. Catch the plug of the other tube between the ring and middle fingers and remove it. During this operation the tubes should be held almost horizontal with the table top so that bacteria from the air are less likely to drop into them. Pass the open ends of the tubes through the Bunsen flame to heat them and then with the sterile platinum loop remove a little of the growth from the culture and carry it into the fresh tube of medium without touching the wall of the tube and rub it over the surface of the medium. Remove the loop, replace the plugs, and sterilize the needle by passing it into the flame and heating it to glowing.

All culture work should be done in a room or compartment protected from dust, draughts, etc.

Pipettes. When it is desired to inoculate definite or large amounts of material or culture, pipettes are indicated. Where the amount of material to be inoculated is to be measured the graduated variety is employed. If it is merely a question of inoculating considerable amounts the volumetric type can be employed. Small capillary pipettes are ideal for the collection and inoculation of various fluids, exudates, etc.

Pipettes should be thoroughly cleaned, plugged with cotton, and sterilized, as described in the chapter on the preparation of glassware, etc.

Glass Spreaders. Glass spreaders are of value in inoculating plate cultures. They are simply pieces of glass rod about 8 inches in length, about 2 inches of the end being bent at right angles to the rest of the rod. These are either wrapped in paper or placed in containers and sterilized. They are used in the following manner: A drop of the material to be cultured is placed on a plate of culture media about midway between the center and edge of the plate. A sterile spreader is then placed in contact with the drop of inoculum, holding the long portion of the rod in an upright position with the angle of the spreader resting on the central portion of the plate. Give the inoculum a few seconds to spread out along the short portion of the rod in contact with the medium and then slowly turn the rod, swinging the part touching the medium once completely around the plate making sure that it remains in contact with the medium as it turns. If culture or material

containing a large number of organisms is plated by this method it is desirable to inoculate a second and third plate with the same spreader employing no inoculum other than that carried over on the spreader from the first plate.

Sterile Swabs. Sterile swabs can frequently be utilized to good advantage for the collection and subsequent inoculation of various material into culture media. If they contain only a small amount of material the swab can be first dipped into a sterile tube of bouillon and then streaked back and forth across a plate of culture medium proceeding from the top to the bottom of the plate. If the swab contains a considerable amount of material, and especially where large numbers of bacteria are suspected or known to be present, it is best to make only one streak across the top of the plate and then spread the material thus placed with a sterile platinum loop.

Pure Cultures. A pure culture of a particular organism may be obtained in several ways. Some pathogenic organisms may be most satisfactorily isolated in pure culture by inoculating experimental animals and making cultures from affected tissues or fluids containing the organism in pure state. Another satisfactory but very tedious way is to employ the Barber single cell isolation method. This involves the use of a special type of apparatus with which a single organism can be drawn into a capillary pipette and subsequently inoculated into a suitable medium. Such method is too complicated and tedious for ordinary purposes. Then, in some cases, certain dyes (gentian-violet, etc.) can be employed to retard growth of organisms other than the one it is desired to isolate. The most common method, however, of obtaining organisms in pure culture is known as the "plating" method. There are two methods of plating in common practice, one is known as the "pour" plate method, the other the "streak" plate method.

Pour Plates. Melt 3 tubes of nutrient agar (not slants but tubes containing about 10 cc. of the medium) and allow same to cool down to 42°C. Inoculate 1 loopful of the culture, or material to be plated, into the first tube moving the loop rapidly back and forth through the medium to remove and mix as much of the inoculum as will readily come off. Now inoculate two loopfuls of the medium from the first tube into the second, and 3 from the second into the third tube. Shake the tubes gently or roll between the palms of the hands to get the inoculated material well distributed but avoid working up a froth. Next flame the lips of the tubes and pour the contents into 3 sterile Petri dishes. Allow the medium to harden and then incubate, inverting the plates so that the moisture will collect in the lids rather than on the surface of the medium. Plates thus inoculated will reveal the organisms growing in more or less separated colonies originating from single

organisms or clumps of organisms. If the original inoculum contained a large number of bacteria the colonies on the first plate will be numerous and possibly so close together that individual colonies cannot be satisfactorily "picked." The second or third plate, however, usually contains colonies sufficiently well separated to study and "pick" for pure culture. If the material contains such enormous numbers of bacteria that all 3 plates are "crowded" with growth then the inoculum must be diluted with broth or saline before plating is again resorted to.

Streak Plates. Streak plates are very convenient to work with and have, in a large measure, replaced pour plates except for bacterial counts. Vari-

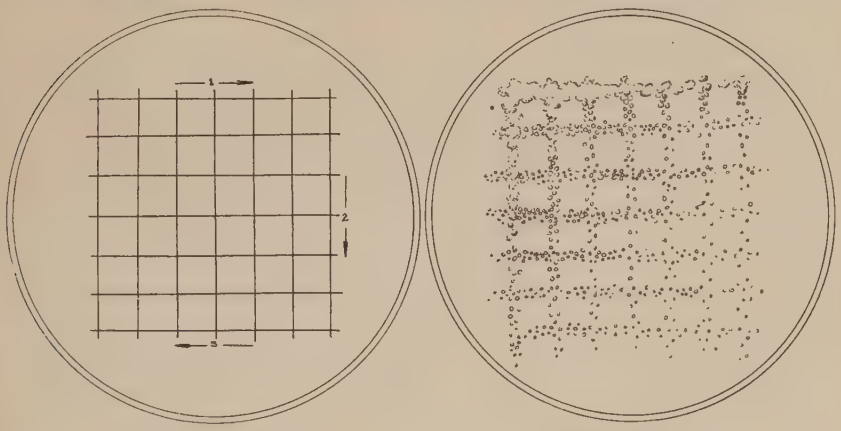


FIG. 14. METHOD OF INOCULATING STREAK PLATES

(*a*) diagram indicating manner in which inoculum is streaked out; (*b*) distribution of colonies.

ous types of culture media are placed in Petri dishes and allowed to harden and are kept on hand for streak plates. This method of plating makes it possible to keep ready for immediate use, not only ordinary agar or gelatin medium, but media containing blood, serum, etc.

In inoculating streak plates a loop, spreader, or swab can be used. The writer has employed the following method of inoculating plates with a platinum loop with highly satisfactory results: Take a loopful of the material to be plated and make a single horizontal streak from left to right across the medium near the outer border of the plate. Sterilize the loop in the flame and then starting at the right end of the streak make vertical streaks at right angles to the first streak proceeding from right to left.

Flame the loop again and then starting at the bottom of the first vertical streak cross all of the vertical streaks and proceed in the same manner upwards towards the first horizontal streak. Figure 14 illustrates this method.

In the first streak the greatest number of organisms will, obviously, be at the beginning, the least at the end. Then by going to the end of the streak with a sterile needle and streaking that portion down, the end of such down streak will contain but very few bacteria.

After well separated or discreet colonies have been obtained they are examined with a hand lens to observe more closely their character and appearance. After one becomes familiar with the colony characteristics of various organisms he can frequently immediately rule out of consideration a large number of organisms of no interest by the appearance of the colony. After locating several colonies which appear typical of the organism one may be looking for, they are "ringed" by making a wax pencil mark around them on that portion of the glass immediately under same. The next procedure is to make a slide preparation for microscopic examination. To accomplish this touch a sterile platinum needle to the colony and mix the minute amount of culture adhering to the needle in a small drop of water or saline soution on a clean slide. This is then smeared out over the middle portion of the slide, allowed to dry and fixed by passing lightly through the flame of a Bunsen burner. The preparation is then stained by Gram's or some other desired method, examined microscopically and, if it is found that but one type of organism is present and that is morphologically like the organism sought, additional cultures on various media are made from the same or similar colonies for further study.

If we are plating to isolate and identify all the organisms present the characteristics of the different colonies are noted, smear preparations are made from each type, stained and examined for purity, and then cultures for further identification made from representative colonies.

Stab Cultures. In inoculating gelatin, Russell's double sugar medium, "stick" agar, etc., a needle rather than a loop is used. The needle is sterilized by flaming, allowed to cool, touched to the inoculum and then stabbed into the medium. In the case of the double sugar medium the needle is first drawn over the surface of the slant and then pushed into the depths of the butt.

Inoculating Culture Media with Anaerobes. Just prior to the inoculation of tubes or flasks of broth layered with oil for the cultivation of anaerobes it is good procedure to heat the contents of such tubes or flasks up to the boiling point to drive off any absorbed oxygen. After allowing the medium to cool down to about 40°C. it is inoculated with the loop, needle or pipette

and immediately placed in the incubator. If a pipette is used care must be taken not to introduce air into the medium.

CULTIVATION OF BACTERIA

Temperature. Next to a satisfactory culture medium, temperature requirements and atmospheric conditions must be taken into account in the artificial propagation of bacteria. As has been previously indicated a large number of the pathogenic bacteria will grow satisfactorily at body temperature so that the ordinary bacteriological incubator is maintained at a temperature of 37.5°C. However, in working with some organisms it is necessary to provide additional incubation facilities to more nearly approximate the particular temperature requirements of same. A number of organisms, especially the moulds, will grow more readily at room and, in some instances, even refrigerator temperature, than in the incubator.

Where gelatin culture medium is employed to determine whether or not an organism is proteolytic such culture must be left at room temperature as the gelatin will liquefy if placed in the incubator. If the organism is fastidious in its temperature requirements and must be grown at incubator temperature, gelatin cultures can be first incubated and later left at room temperature to determine whether or not they will solidify. If the particular bacterium belongs to the proteolytic group the gelatin will not solidify. Where it is possible to grow the organism in gelatin medium at room temperature such is preferable as the nature of the growth, rapidity of liquefaction, etc., can be observed.

Cultivation of Aerobes. In growing aerobes and facultative anaerobes one merely inoculates the organism on or into suitable medium and places the same under proper temperature conditions. In dealing with strict aerobes conditions must be such that the organisms can get an ample amount of oxygen. With M. tuberculosis grown on broth the inoculum must be floated on the surface as the organism will not grow below the surface.

Cultivation of Anaerobes. The propagation of strict anaerobes and particularly their initial isolation has given bacteriologists trouble for many years. A great amount of work has been done with a view to improving anaerobic methods. The result has been the advocation of a number of methods described for the culture of anaerobes. Some of these methods are satisfactory when used with one or more types of anaerobes but not with others. Some work well after an anaerobe has been isolated in pure culture but are discouraging when it comes to primary isolation. Of the various methods which follow the author has had more general satisfaction with the Brown-jar method than with any of the others. The other methods are

valuable under certain conditions and with certain organisms, but for general purposes the Brown-jar method is strongly advocated.

Oxygen Exclusion Method. The simplest method of growing organisms under anaerobic conditions is to employ culture tubes two-thirds to three-fourths full of glucose agar, gelatin, or other dense, solid medium which immediately before use is boiled to exhaust absorbed oxygen and then rapidly cooled by placing in the ice-box or in ice water to prevent absorption of more oxygen. Such media when inoculated deeply by means of a platinum needle usually give satisfactory results. Fluid media layered with liquid petrolatum or vaseline and heated and cooled in the manner described, likewise give satisfactory results. The vaseline seal is better than the liquid petrolatum as air will gradually penetrate through the oil.

This method can be used for pure culture isolation where relatively few organisms are inoculated into the tube. The procedure is known as the "shake culture" method and is carried out in the following way: Melt the desired number of tubes of agar or gelatin and leave in a water bath of boiling water for 10 or 15 minutes to drive off oxygen. Allow the medium to cool down to 42° to 40°C., inoculate, and then gently shake the tubes to get the organisms well distributed. Solidify rapidly by placing the tubes in ice water. After incubation, isolated colonies may be seen scattered through the medium. The outside of the tube can then be sterilized with alcohol and carefully broken and the medium placed in a large sterile Petri dish and representative colonies "fished" and transferred to fresh medium.

While this method is very commonly employed for the propagation of pure cultures of anaerobes, it is obviously not very satisfactory for the initial isolation of anaerobes from material containing a variety of bacteria. Methods which permit of the use of plate cultures are much to be preferred.

Oxygen Exhaustion Method. The method that would naturally occur to one is that wherein cultures are placed in jars from which the air has been exhausted by a vacuum pump. For this purpose a Novy jar or desiccator is employed. A vacuum pump attached to the water faucet or, better, a motor-driven pump, can be employed. The jar must be perfectly air-tight around stop-cock and lid. A thin film of petrolatum should be placed between adjoining pieces. This method is not very satisfactory. In the first place the medium drys very rapidly and is thus rendered less suitable for bacterial growth. Then the surface tension obtaining under such vacuum may interfere with growth. Finally, it is almost impossible to exhaust the average jar and maintain it absolutely free of oxygen.

Displacement Method. The normal atmosphere in a Novy jar can be replaced by hydrogen. Such gas can be generated through the use of a

Kipp generator or it can be supplied direct from a cylinder. After the plates or other cultures have been put into the jar and the lid securely fixed in place, it is coupled to the oxygen supply by a piece of rubber tubing running from the generator or cylinder to the intake of the jar. The oxygen is then permitted to flow down through the intake of the jar thus forcing the air out through the outlet. The flow of hydrogen through the jar is continued for about 10 minutes when the stop-cocks are closed and the jar placed in the incubator.

Where the hydrogen is generated with zinc and sulphuric acid in a Kipp generator the usual procedure is to use common, commercial zinc and a 10 per cent solution of sulphuric acid. Hydrogen generated in this manner contains impurities which should be removed before passing it into the culture jar. Ample purification can be accomplished by passing the gas through a series of wash bottles interposed between the generator and jar. Ordinarily 3 wash bottles are employed. The first holds a 10 per cent lead acetate solution to remove traces of hydrogen sulphide, the second a solution of equal parts of pyrogallic acid and sodium hydroxide dissolved in about 10 volumes of water, to absorb traces of oxygen coming over, and the third a 10 per cent silver nitrate solution to remove any arsenide of hydrogen (AsH_3).

In handling hydrogen it should always be borne in mind that such gas is inflammable and when combined with oxygen is highly explosive. Where hydrogen is being permitted to flow through a jar to displace the normal atmosphere the flow should not be permitted to go on over a protracted period, particularly in a small, poorly ventilated room. While the amount of hydrogen liberated in the average room during the displacement process with a Novy or similar jar, is not *particularly* dangerous, the safest procedure is to connect a piece of rubber tubing to the outlet of the jar and pass the other end out of the window.

This method of propagating anaerobes gives satisfactory results in many instances but is not infallible.

Alkali-Pyrogallic Acid Method. This is a widely used method for the propagation of anaerobes. The basis of the method lies in the fact that pyrogallic acid when placed in an alkaline solution will absorb large quantities of oxygen. The culture tubes or plates can be placed in a wide mouth jar or better in a desiccator or Novy jar. If a desiccator is used the porcelain plate serves to keep cultures above the chemical mixture. If an ordinary or Novy jar is employed a rack must be used to keep the plates out of the mixture of alkali and pyrogallic acid.

Use about 1 gram of pyrogallic acid for each 100 cubic centimeters of air

space. Place the pyrogallic acid in the bottom of the desiccator or jar and then put the cultures in. Next run in rapidly on the pyrogallic acid 10 cc. of a 10 per cent aqueous solution of NaOH or KOH for every gram of pyrogallic acid used. Quickly close the jar and see that it is air tight. The alkali-pyrogallic acid mixture, which at first is of a light brown color, becomes a dark brown as oxygen is absorbed.

Various modifications of the alkali-pyrogallic acid method of cultivating anaerobes have been described from time to time.

Buchner's Method. In dealing with small numbers of tube cultures a very simple and convenient anaerobic method is that first utilized by Buchner.

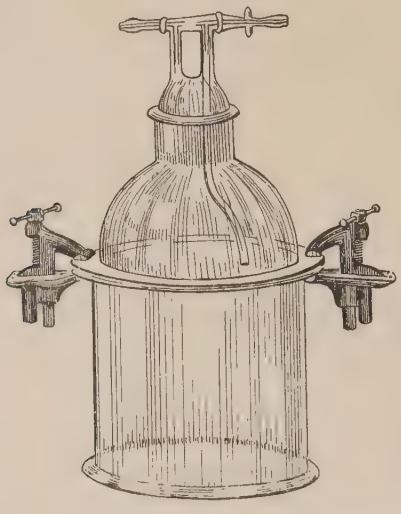


Fig. 15. Novy Jar for Use in Cultivating Anaerobes in an Atmosphere of Hydrogen

Provide several rubber-stoppered tubes of such size that the culture tubes used can be placed in same. Place in the bottom of the larger tube a short piece of glass rod which will serve to keep the smaller culture tube elevated in the larger tube so that the former is not soiled by the chemical mixture. Place in the bottom of the larger tube a small amount of pyrogallic acid. When everything is ready run in a little 25 per cent NaOH or KOH solution, quickly place the tube containing the inoculated medium in the larger tube and securely stopper the latter with the rubber stopper. Paint the joint with melted paraffin and it is ready to go in the incubator.

Before the smaller tube is inoculated, if the medium is such that it can be

heated, it should be boiled for a few minutes to drive off any absorbed oxygen it may contain, rapidly cooled, inoculated, and placed in the larger tube as described above.

Wright's Method. After making a deep stab inoculation into glucose-agar or gelatin the absorbent cotton plug of the tube is flamed and pushed into the tube so that the upper portion of the plug is $\frac{1}{2}$ to $\frac{3}{4}$ inch below the lip of the tube. Next a small amount of pyrogallic acid is placed on top of the cotton plug and this is followed with about 2 cc. of a 10 per cent solution of NaOH. A rubber stopper is then quickly placed tightly in the tube to make it air tight.

Zinsser's Method. In this method two crystallizing dishes are used, one approximately the diameter of the regular Petri, the other just a trifle larger so that the smaller will fit into the larger with about one-fourth inch space between the walls of the two. These dishes are sterilized in pairs in the same manner as Petri dishes.

If ordinary agar is the culture medium employed, tubes of same are melted, allowed to cool to 45°C., inoculated, and poured into the smaller of the crystallizing dishes and covered with the larger, the procedure being identical with that followed in making pour plates. After the agar has hardened the dish containing it is carefully inverted over a wet piece of filter paper to temporarily protect it from contamination.

In the center of the larger dish there are placed about 3 grams of pyrogallic acid. The smaller dish containing the culture medium is now inverted over the dry pyrogallic acid and a 5 per cent solution of NaOH is run down as rapidly as possible between the walls of the two dishes until the bottom dish is about one-half full. Quickly place a layer of paraffin oil over the surface of the alkali solution in the space between the walls of the two dishes, thus sealing it from the air. It is very important that as little time as possible elapse between the adding of the alkali solution and the placing of the paraffin oil. An assistant should be ready with a pipette filled with paraffin oil ready to run it in the instant the addition of the alkali is completed.

In the utilization of the above method if it is desired to employ types of solid media which can not be melted after preparation, such media can be put up in the smaller crystallizing dishes and handled the same as streak plates.

Tissue Enrichment Method. Various investigators (Tarozzi, Th. Smith, Noguchi, etc.) have called attention to the fact that small pieces of sterile tissue, placed in the bottom of tubes of solid or liquid culture medium promote the growth of anaerobes. Sterile pieces of kidney or spleen from a rabbit or guinea pig are commonly used for such purposes.

Where this method is resorted to it is best to have tubes two-thirds to three-fourths full of medium and where the same is fluid a layer of paraffin oil is desirable.

Krumwiede and Pratt's Method. In this method the bottom portions of Petri dishes are placed open side up in their covers, wrapped and sterilized. Tubes of melted agar are inoculated as practiced for pour plates. The inoculated agar is then poured into the cover of one of the sterile Petri dishes and the bottom immediately placed in the cover, open side up, taking care to see that there are no air bubbles between the medium and the dish. After the agar has hardened melted paraffin is run over the exposed edge to prevent drying and contamination of the medium. Care should be taken to use tubes of agar containing just a sufficient quantity to fill the dish cover without marked overflow when the bottom is put in place.

After incubation the halves of the plates can be carefully pulled apart and representative colonies "fished." Usually the agar remains intact in the lid of the Petri dish when top and bottom are separated. If part does adhere to the bottom dish it can be carefully removed with a spatula and returned to the cover. A sterile Petri dish lid of larger diameter or a crystallizing dish can be inverted over the lid containing the culture to prevent contamination.

Brown's Modification of McIntosh and Fildes' Method (Brown-jar Method). McIntosh and Fildes,[1] working with anaerobes of war wounds, adopted a modification of the method first suggested by Laidlaw,[2] deoxygenating atmosphere by combustion with hydrogen. They utilized "capsules" of palladinized asbestos wool in culture tubes and jars into which they subsequently passed hydrogen. The "capsule" is heated just before use and as hydrogen is permitted to slowly enter the tube or jar it is oxidized through the agency of the palladinized asbestos with the formation of water. The process continues until all the free oxygen in the jar has been exhausted.

Several methods of utilizing this principle for the cultivation of anaerobes have been described. The one used by the author with excellent results is that described by Brown,[3] the apparatus used being commonly referred to as the "Brown-jar." A round type museum jar about 15 inches in height and 6 inches in diameter can be employed. It is necessary, however, to drill a hole of about $\frac{1}{2}$ inch diameter through its glass lid about 2 inches from its edge. The next step is to prepare the "spool" containing palladinized asbestos around a spiral of nichrome resistance wire which constitutes a

[1] McIntosh and Fildes, P., Lancet, 190, 1916.
[2] Laidlaw, P. P., Brit. Med. Jour., i, 497, 1915.
[3] Brown, Jr. Exper. Med., xxxiii, 677, 1921.

heating unit to "activate" the palladinized asbestos. A piece of glass tubing 10 or 12 inches long and 6 or 7 mm. in diameter is sealed at one end by heating it in a flame. Using a fine blue flame, such as can be obtained with an ampule-sealing blow torch, the tube is then heated about 3 inches from the sealed end and when it starts to soften a small hole is blown in the heated portion by placing the cool end of the tube in the mouth and blowing until a small glass bubble forms and bursts. By careful heating, the ragged portions of the bursted bubble are melted down and we have a tube with a small hole in it. Allow the tube to cool and then with a sharp file cut off the sealed end to within one inch of the hole. Now seal off the other end of the tube and allow it to cool. Slip a short piece of tight-fitting rubber tubing over the open end of the tube allowing it to come down sufficiently far to cover the first hole made. Now heat the tube about 2 to $2\frac{1}{2}$ inches below the first hole and proceed to make a second in the same way as the first was made. Allow the tube to cool and cut it off about one inch beyond the second hole. We now have a piece of glass tubing about 4 to $4\frac{1}{2}$ inches long with a small hole about 1 inch from each end.

Have ready a piece of 24 gauge nichrome wire about 10 or 12 inches long on each end of which about a foot of 16 gauge copper wire has been securely soldered. A piece of very thin asbestos board is soaked in water and molded around the piece of glass tubing between the two holes to protect the glass when the wire is heated. One end of the copper wire is passed in the end of the tube and directed up through the first hole and pulled until it and the nichrome wire have been brought through. The nichrome wire is spiralled around the asbestos-covered tube, regulating the distance between the spirals so that the nichrome wire is evenly distributed between the two holes. The copper wire is then passed through the second hole and out the end of the tube pulling it sufficiently tight to hold the spiralled nichrome wire in place. By bending the copper wire coming out of each end of the tube at an abrupt right angle, tension is maintained and the spiral of nichrome wire remains in place.

Two asbestos washers of about $\frac{3}{4}$ inch diameter are made of thin asbestos board and one slipped on each end of the tube up to the hole where the wire comes through. A fiber spigot washer of the same size is then placed on each end of the tube up against the asbestos washer. This second washer is rigid and serves as a support. A piece of fine mesh copper gauze is then fashioned around the rod extending it about $\frac{3}{4}$ inch over the ends of the washers and keeping it about the same distance from the tube along the central portion as the washers keep it away at the ends. Short pieces of copper wire 5 or 6 inches in length and a small quantity of palladinized

asbestos should be in readiness. The space between the tube and the copper gauze is filled with the asbestos and the gauze securely fastened in place by twisting several pieces of the copper wire around the "spool." A piece of the copper wire should be drawn around the gauze where it laps over the washers so that it is drawn down snugly in place. If there is an excess of the gauze at the ends it can be trimmed off with a pair of scissors.

The palladinized asbestos is prepared as follows: Place 1 gram of clean, finely divided asbestos wool in a porcelain evaporating dish and add to it 6 cc. of a 10 per cent solution of palladium chloride to which a little HCl has been added to favor solution, stirring the wool with a glass rod as the fluid is added. The wool is then placed in the incubator or hot air oven and allowed to slowly dry. After it has dried it is carbonized in a yellow flame. When well coated with soot the palladium is reduced in the asbestos by heating it to glowing through the use of a flame and blow pipe or by exposing it to the direct flame of the Bunsen burner. The wool is now ready for use.

After completing the "spool" it is attached to the lid of the jar. The two ends of the copper wire are brought through the hole in the lid letting the spool hang down several inches. A piece of glass tubing bent at right angles, to serve for the hydrogen intake, is then passed into the hole between the two copper wires and melted sealing wax is used to fill the space about same. It is important that the copper wires be well separated in order to avoid a short circuit when current is passed into the spool. A generous amount of sealing wax should be used to fill in the hole leaving a surplus of wax around the hydrogen intake tube and wires on both the inside and outside of the lid. The free ends of copper wire can be provided with connections for coupling to the current leads or simple loops can be made to attach to

In order to insure proper heating of the nichrome wire in the spool when current is applied, an arrangement is made whereby two 100 to 150 watt lamps are coupled in series with the heating unit of the "spool." All that is needed for this is a length of ordinary electric lamp-cord with a plug attached to one end and a small piece of board on which is mounted two lamp receptacles. The two wires composing the lamp cord are separated at one end for a short distance. One of these is fitted with a terminal to connect direct with one of the wires leading out of the Brown-jar from the spool. The other wire is connected on one side of one of the lamp receptacles. A short piece of wire is then used to make a connection from the other side of the receptacle to the second socket. Finally a piece of single wire is then attached to the remaining connecting joint on the second receptacle and a terminal is fixed in the free end of the wire to be attached

to the second wire of the Brown-jar. Two 100 to 150 W. carbon filament lamps are used in each receptacle.

The Brown-jar is used in the following way: First of all a small cotton-stoppered test tube of sterile, alkaline, dextrose bouillon containing just enough methylene-blue to give it a distinct blue color, is attached to the inside wall of the jar to act as an indicator for the presence or absence of oxygen. This tube can be put in place by several pieces of adhesive tape or it can be mounted in a little plastercine and be made to adhere to the side of the jar.

A towel or a layer of non-absorbent cotton is then placed in the bottom of the jar. After the culture tubes or plates have been put in the jar $\frac{1}{4}$ to $\frac{1}{2}$ inch of plastercine (molding clay) is placed evenly around its edge. A similar amount is placed around the edge of the lid and the same placed

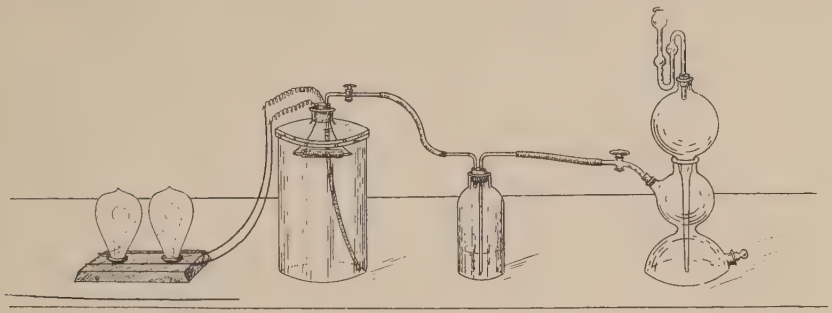


FIG. 16. BROWN-JAR FOR THE CULTIVATION OF ANAEROBES

lightly on the jar without pressing it down. The jar is coupled to the hydrogen generator (using wash bottles) and to the electric current but the current is not turned on immediately. Open the hydrogen stop-cock and allow the gas to bubble slowly into the jar through the glass tube intake to which a piece of rubber tubing has been affixed so the gas will be liberated in the bottom of the jar. As the lid has been placed only lightly on the jar the hydrogen forces a large percentage of the normal atmosphere from the jar through the leaks around the lid. The flow of gas is continued just sufficiently long to insure mechanical replacement of most of the oxygen. While this is occurring it will be noted that the "spool" gets warm and moisture starts to collect on the sides of the jar. If the "spool" has been in use some time the reaction is not near so active as with a new "spool" and the electric current must be soon resorted to for the purpose of warming up the palladinized asbestos. With new "spools" it is often only necessary to use the current toward the end of the procedure.

After running the hydrogen through sufficiently long to displace the normal air in the jar the lid is then pressed firmly down on the jar stopping the leakage of gas around the lid. Now turn on the current. If the jar is tight the hydrogen flow will now be slowed up, coming over only as fast as it can be combined with the oxygen present through the agency of the palladinized asbestos. This rate varies considerably depending upon how much the apparatus has been used. If the spool is relatively new it will be more rapid than with an old spool. If the reaction is taking place rapidly, the electric current should be turned off and the flow of hydrogen cut down so that it bubbles in slowly. When the process slows up the current can be turned on. After the jar has been in operation over a period of time sufficient to have exhausted most of the free oxygen present (indicated by only a very occasional hydrogen bubble passing through the system) the regular clamp which is used with the specimen jar should be put in place as an added safeguard against leakage.

After the jar stops taking hydrogen (usually within 1 or 2 hours) a Mohr pinch-clamp is placed on the rubber tubing going from the jar to the gas generator and after tightly screwing up the clamp the jar is disconnected from the gas apparatus and electric current and placed in the incubator. Complete decolorization of the methylene blue tube, indicating anaerobiosis, usually occurs overnight.

In place of using a specimen jar a jar similar to that shown in the illustration can be purchased from dealers in laboratory equipment. The lid of such jar has an opening into which a rubber stopper is fitted, the hydrogen intake tube and electric wires passing through the stopper. The intake tube is fitted with a glass stop-cock which is an advantage over the rubber tubing and Mohr pinch-clamp.

This method provides an atmosphere saturated with moisture, free of all oxygen, and gives but a slightly changed surface tension. In using this method there are several precautions which should be emphasized with a view to preventing possible explosions. First of all make sure that the wires coming away from the spool are intact each time before using the jar. Next be sure to always replace mechanically most of the air in the jar by allowing the hydrogen to flow slowly through it *before the lid is clamped down.* Do not allow the spool to reach a red heat as may happen with a new spool where hydrogen is run in rapidly. Cut down the flow of hydrogen, if necessary, turning the stop-cock entirely off for a few minutes to allow the spool to cool down a little. Make sure that there are no loose electric connections and use current only when necessary. It is frequently necessary to use current only towards the end of the process. Do not clamp on the lid

until the jar has been operating awhile. If most of the atmosphere has been replaced mechanically, as described, and the lid is not clamped down immediately, any overlooked defect in the spool and its wires which might result in a spark before the last of the oxygen was exhausted, would cause only a very minor explosion. When handling the jar while it is in operation take the precaution to cut the current off during such handling.

Growth of Anaerobes in Symbiosis with Other Bacteria. In mixed culture it frequently happens that certain anaerobes and microaerophiles are able to propagate through the marked oxygen-consuming property of the organism or organisms they are associated with. This principle can be put to practical use in cases where it is desired to cultivate bacteria (microaerophiles) which thrive best at a reduced oxygen tension. Cultures of such organisms placed in tight jars with cultures of bacteria which consume large quantities of oxygen (B. subtilis for example) will grow satisfactorily.

Propagation of Bacteria in Atmosphere Rich in CO_2 and Low in Oxygen. It has been shown by Wherry and Erwin,[4] Cohen and Fleming,[5] Huddleson,[6] Buck,[7] etc., that the growth of certain bacteria is favored when the cultures are incubated in an atmosphere which has been replaced to the extent of approximately 10 per cent with CO_2. In the initial isolation of Alcaligines abortus (Bang) from tissues or animal fluids this is a highly valuable method. If this method is not employed, then, to obtain initial isolation of such organism, some other method of growing the cultures under conditions of partial anaerobiosis must be resorted to.

Propagation of Bacteria for Detection of Gas-producing Properties. In propagating bacteria to determine whether or not they are capable of producing gas from various fermentable substances, medium containing the desired carbohydrate or other fermentable agent is distributed into fermentation tubes. Several types of such tubes are available. The Smith fermentation tube is a type frequently employed and when used the amount of gas production can be more satisfactorily approximated than with the Durham tube which is used on a rather large scale. The Durham tube consists simply of an ordinary test tube into which is placed a smaller tube or phial in an inverted position. Where quantitative results are of little importance Durham tubes are entirely satisfactory and can be handled somewhat more conveniently than the Smith tubes. In the Smith tube the gas is trapped in the arm of the tube forcing the medium it displaces

[4] Wherry and Erwin, Jour. Inf. Dis., 22, 1918, 194.
[5] Cohen and Fleming, Jour. Inf. Dis., 23, 1918, 337.
[6] Huddleson, Mich. Agric. Col. Exp. Sta. Bul. No. 49, November, 1920.
[7] Buck, Jour. Agric. Research, vol. xxix, no. 12, December 12, 1924.

down into the bulb. With the Durham tube gas collects in the upper portion of the inverted small tube or phial.

It is usually customary to place a small amount of some type of indicator (phenol red, Andrade, litmus, etc.) in the various fermentable media to determine whether or not acid is produced by organisms inoculated into same.

CHAPTER VII

Microscopic Study of Bacteria; Stains and Staining Methods

Unfortunately bacteria cannot ordinarily be identified by merely studying their appearance with the aid of the microscope. Morphology is only one link in the chain of evidence which must be gathered before an organism can be identified. Thus, morphology, physiology, cultural characteristics, staining properties, biological reactions, and pathogenicity all come in for consideration in the identification of bacteria. This does not mean that all organisms must be put through all and the same series of examinations before identification is possible. Some bacteria can be identified after a comparatively brief examination or study by which certain definite characteristics peculiar to the organism are brought out. On the other hand, a vast amount of work must be done with some forms to differentiate them from closely related organisms and definitely identify them.

In order to study bacteria microscopically preparations from the cultures or material to be examined must be made on glass slides or cover-glasses. These preparations may be dried and stained by various methods or they may be examined in a moist, unstained state. Examination of moist, unstained preparations will give information as to the number of organisms present and their size and will indicate whether they are motile or non-motile. Moist specimens known as "hanging drop preparations" are commonly made to determine whether or not an organism is motile.

Hanging Drop Preparation for the Determination of Motility. For making a hanging drop preparation a special slide with a concavity ground in it is employed. With a tooth pick, match stem or small swab place a film of vaseline around the concavity in the slide. If the culture or other material to be examined, is in a fluid state place a drop on the center of a clean cover-glass. If a culture on solid medium is to be examined emulsify a small amount of same in a drop of distilled water on the cover-glass. Now quickly invert the cover slip and place it over the concavity in the slide so that the drop is suspended over the center of the depression without coming in contact with the slide. The edges of the cover-glass are pressed down firmly in the petrolatum to prevent the slip from moving and to prevent evaporation of the drop.

When examining a hanging drop preparation to determine whether or not an organism is motile care must be taken not to confuse "Brownian

movement" with true motility. Brownian movement is so called because it was first described by a botanist named Brown. Brownian activity is a molecular movement manifested not only by living bacteria but by dead organisms, finely divided particles of organic matter, etc. It is a sort of trembling or vibrating, back and forth movement, the organism or particles not actually leaving a given field as a result of such movement. On the other hand, in true motility, produced by the flagella of the bacteria, the organisms do not remain in a definite position but are seen to move about the field in different directions, the rate of movement being very rapid with some species and sluggish with others. One should, of course, not be mislead by movements due to currents in the preparation. Under such conditions everything in the "stream" moves in the same direction and at about the same rate of speed. There should be no difficulty in eliminating movement due to currents. A final method for differentiation between Brownian

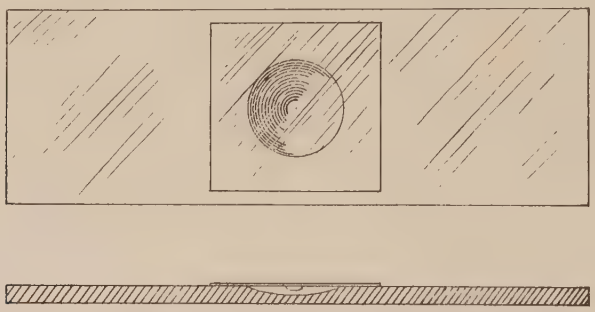


Fig. 17. Hollow Ground Slide for Hanging-Drop Preparation

activity and true motility is to add a little formalin (3 per cent) or phenol (5 per cent) solution to some of the culture and then compare with the untreated specimen. True motility is, obviously, absent after the bacteria have been killed by the antiseptic. Brownian movement, on the other hand, is not interfered with.

For motility examinations young bouillon cultures (about 18 hours old) usually give best results. The microscope should be placed where it will receive the least vibration and the examination made with the high power dry objective and reduced light. Care must be taken in focusing the objective to prevent breaking the thin cover-glass.

Stained Preparations. For a more detailed study of the structure of bacteria dried film or "smear" preparations which have been stained are utilized. Staining not only brings out the structure of the microbe better, but in addition serves as an aid in its identification. In other words, the

way certain organisms stain or fail to stain with certain dyes and methods is a large factor in identifying bacteria.

The slides and cover-glasses used in making bacterial preparations for microscopic examinations should be cleaned as described in the chapter on the preparation of glassware. Just before use they should be passed through a Bunsen flame to burn off any traces of grease. In flaming cover-glasses pass them back and forth through the flame rapidly so as not to melt them.

To make a preparation from a culture on solid medium for microscopic study place, with a platinum loop, a small drop of distilled water on a slide or cover-slip held with a pair of Stewart or similar forceps. Sterilize the loop in the flame and allow it to cool. With the sterile loop carefully pick up a very minute amount of the culture growth. Do not endeavor to pick up a large amount. An amount so small as to be almost indiscernible on the loop is sufficient. If too large an amount is taken the organisms will be so numerous and close together on the slide as to materially lessen the value of the preparation. Now mix the bit of culture in the drop of water on the slide and spread it around the middle third of the slide's surface. The preparation is allowed to dry in the air or it may be held high over a Bunsen flame until it dries. With fluid cultures a loopful of the undiluted culture is streaked back and forth across the center of a clean slide and allowed to dry. The film or "smear" must now be "fixed" otherwise it will be washed off during the staining process. The fixing is commonly accomplished by passing the slide, film side up, rather slowly through the flame several times. There are other methods of "fixing" by chemical reagent which will be indicated subsequently.

After the preparation has been fixed it is ready to be stained by one of the various methods employed in bacteriology. The choice of the method depends upon the organism, if known, or the staining characteristic to be tested for where the microbe is being examined to determine its action towards a certain stain or method of staining.

The outstanding example of staining reactions in the identification of bacteria is found with *Gram's method*. Bacteria are divided into two major groups according to whether they retain the first stain used in the Gram method, following subsequent decolorizing treatment, or become decolorized and take the counterstain. Bacteria which retain the initial stain are spoken of as *Gram-positive* organisms while those which become decolorized and take the counterstain are referred to as being *Gram-negative*. While it is true that there are a few organisms which are irregular, being Gram-positive one time and Gram-negative at another, this method is highly important in the differentiation of a large number of bacteria.

When an organism stains well with the ordinary aniline dyes methylene-blue (Loeffler) or carbol-fuchsin (Ziehl-Neelsen) can be used with good results, these being the two most common stains found in bacteriological laboratories. Ordinarily such stains are best applied in a diluted form. Cover the preparation on the slide or cover-glass with distilled water and then drop into this several drops of the stain from a dropping bottle. Tilt the preparation back and forth to distribute the stain through the water. After staining about 2 minutes, wash with tap water and dry between several small squares of filter paper.

If the preparation is made on a slide a drop of Canada balsam can be placed on the stained "smear" and a clean, thin cover-glass dropped on it and lightly pressed down to spread the balsam and release air bubbles. If the preparation is not to be kept it is not necessary that a cover glass be used. The specimen can be studied by placing a drop of cedar oil directly on the film and examining it with the immersion oil lens. Where the preparation has been made on a cover-glass a drop of Canada balsam is placed on a clean slide and the cover-slip dropped on it, with the film down, and pressed to spread the balsam and release air bubbles.

The staining characteristics and qualities of the different organisms met with in veterinary medicine are taken up under the various organisms considered.

STAINS AND STAINING METHODS

Formulae and methods for the preparation and use of stains commonly employed in the study of bacteria dealt with in veterinary medicine follow.

Alkaline-Methylene-Blue (Loeffler)

Saturated alcoholic solution of methylene-blue	30 cc.
Potassium hydrate (1:10,000 aqueous solution)	100 cc.

(Approximately 7 per cent methylene-blue in alcohol makes a saturated solution)

Carbol-Fuchsin (Ziehl-Neelsen)

5 per cent aqueous solution of phenol	100 cc.
Alcohol (95 per cent)	10 cc.
Basic fuchsin	1 gram

Another simple way of preparing carbol-fuchsin solution is to add 10 cc. of a saturated alcoholic solution of fuchsin to 100 cc. of a 5 per cent solution of phenol. This is slightly weaker than that prepared by the formula given above.

Gram's Staining Method. This method is based on the fact that certain bacteria (Gram-positive organisms), when stained with gentian-violet and subsequently treated with iodine solution, are not decolorized by alcohol or acetone and, hence, will not take a counter-stain. Gram-negative organisms, on the other hand, are decolorized by alcohol or acetone following staining with gentian-violet and treatment with iodine and take the counterstain.

The exact nature of the Gram reaction is not definitely understood. There has been considerable discussion on the subject and a number of investigators have endeavored to clear the situation but as yet the matter has not been finally settled. An explanation accepted by many is that when the gentian-violet and iodine are brought together in a Gram-positive organism an alcohol and acetone insoluble compound (iodin-pararosanilin-protein compound[1]) is formed. The factor necessary to bring about the formation of this insoluble compound is absent in Gram-negative organisms.

The technic of the Gram method has been the subject of many modifications. The original method specified the use of aniline-gentian-violet, Gram's iodine solution and alcohol for decolorizing. The various solutions are prepared as follows:

Aniline-Gentian-Violet

Aniline oil water	75 cc.
Saturated alcoholic solution of gentian violet	25 cc.

The aniline oil water is made by adding 2 cc. of aniline oil to 100 cc. of distilled water and shaking same vigorously for 5 or 10 minutes. This is then filtered through paper several times. Aniline-gentian-violet will keep only a few days.

Gram's Iodine Solution

Iodine	1 gram
Potassium iodide	2 grams
Distilled water	300 cc.

In carrying out the staining cover the fixed film with the aniline-gentian-violet and allow it to act from 3 to 5 minutes. Pour off the excess of stain. Flood slide or cover-glass with Gram's iodine solution and allow it to act 2 or 3 minutes. Rinse off the iodine. Decolorize with 95 per cent alcohol until no more gentian-violet comes out of the preparation. Decolorization should be promptly discontinued when this point is reached. Wash in water and counterstain 1 or 2 minutes with an aqueous solution of safranin, dilute fuchsin or Bismarck brown.

[1] Chemical Pathology, Wells, W. B. Saunders Co., Philadelphia, Pa.

For the preparation of safranin use 10 cc. of a saturated alcoholic solution of safranin in 90 cc. of water. In place of safranin a 1:10 dilution of Ziehl-Neelsen's carbol-fuchsin may be used. If Bismarck brown is employed as the counterstain it can be prepared by adding 200 mgm. of the dye to 100 cc. of boiling water, allowing it to cool and filtering.

In place of using the aniline-gentian-violet for Gram staining a number of bacteriologists employ the gentian-violet solution recommended by Weigert. This is made up in two fractions which, when kept separate, do not deteriorate. For use small quantities of the two stock solutions are mixed in proper proportions, the amount made up being just sufficient to meet several days' need.

Weigert's Gentian-violet

Solution "A"

Gentian-violet	2 grams
Aniline oil	9 cc.
Alcohol (95 per cent)	33 cc.

Solution "B"

Gentian-violet	2 grams
Distilled water	100 cc.

For use mix 1 cc. of solution "A" with 9 cc. of solution "B" and filter. The mixed stain will keep not longer than about two weeks.

Another modification of the aniline-gentian-violet originally employed by Gram is that recommended by Stirling. It is prepared in the following manner:

Stirling's Aniline-gentian-violet. Place 2 cc. of aniline oil in 10 cc. of 95 per cent alcohol and shake vigorously. To this add 88 cc. of distilled water and again shake. Place 5 grams of gentian-violet in a large mortar. Slowly add the aniline solution grinding the dye into same as added. Allow the preparation to stand overnight and then filter. This solution stains rapidly and intensely and possesses excellent keeping qualities.

The author employs Stirling's aniline-gentian-violet in routine Gram staining using the following technic:

Technic of Gram's Method.

1. Fix the preparation by heat or with methyl alcohol.
2. Stain with Stirling's gentian-violet one minute.
3. Wash lightly with water.
4. Cover preparation with Gram's iodine solution and allow it to act one minute.

5. Pour off excess of iodine solution and decolorize with acetone until no more stain comes out of the film.

6. Wash with water.

7. Counterstain with 10 per cent aqueous solution of safranin for one minute.

8. Wash lightly in water, dry and preparation is ready for examination.

It will be noticed that acetone rather than alcohol is employed for decolorization. It is believed that better results are obtained where such substitution is made.

Some workers prefer carbol-gentian-violet for Gram staining. This is prepared by adding 10 cc. of a saturated alcoholic solution of gentian-violet to 100 cc. of 5 per cent phenol solution.

Neisser's Method for Staining Polar Bodies. This method of staining, frequently used for the staining of the diphtheria organism, is well adapted for bringing out polar staining in organisms of the hemorrhagic septicemia group.

1. The film or smear preparation is fixed and then stained for 5 seconds with the following solution:

Neisser's Stain

Methylene-blue	0.10 gram
Alcohol, absolute	2.00 cc.
Acetic acid, glacial	5.00 cc.
Distilled water	100.00 cc.

The methylene-blue is placed in a test tube and the alcohol added to it. After it has dissolved pour it into a flask containing the water to which the acid has been added. Rinse out any of the dye solution remaining in the test tube by pouring a little of the mixture into the tube and then pouring it back into the flask.

2. Wash the preparation in water.

3. Counterstain 5 seconds with a 0.40 per cent aqueous solution of Bismarck brown. The Bismarck brown solution is prepared by adding 0.40 gram of Bismarck brown to 100 cc. of boiling water. Filter if necessary. Allow it to cool before using.

Staining Acid-fast Bacteria. Organisms of the so-called acid-fast group, because of the waxy or fatty substance which they contain, must be subjected to special technic to satisfactorily stain them. They are designated "acid-fast" because after they are once stained by a satisfactory method they are not decolorized by acids. Some of them resist both acid and alcohol while others are decolorized by the alcohol.

Ziehl-Neelsen's Method.

1. Fix the preparation by heat.
2. Cover slide or cover slip with Ziehl-Neelsen's carbol-fuchsin and steam (without boiling) for 3 to 5 minutes.
3. Wash in water.
4. Decolorize with 95 per cent alcohol to which 3 per cent hydrochloric acid has been added until no more color comes out.
5. Wash in water.
6. Counterstain 1 minute with Loeffler's methylene-blue solution.
7. Wash in water, dry and mount.

Nitric or sulphuric acid in 5 per cent solution may be substituted for the HCl.

Hermann's Method. By this method acid-fast organisms are stained a violet color which they retain when treated with acid. Two stock solutions are kept on hand and these are mixed in small amounts immediately before use.

Solution "A"

Crystal violet	3 grams
Alcohol (95 per cent)	100 cc.

Solution "B"

Ammonium carbonate	3 grams
Distilled water	300 cc.

Dilute one part of "A" with 3 parts of "B."

The technic of the method is as follows:

1. Prepare and fix preparation.
2. Flood preparation with above stain and steam gently for 3 minutes.
3. Decolorize with 10 per cent nitric acid.
4. Rinse well in 95 per cent alcohol.
5. Rinse in water.
6. Counterstain 1 minute with Bismarck brown.
7. Wash, dry and mount.

Gabbett's Method. In this method the preparations are fixed and then stained with steaming carbol-fuchsin in the manner described above. The decolorizing and counterstaining is accomplished in one operation through the use of the following stain which is allowed to act for 1 or 2 minutes:

Methylene-blue	1 gram
Sulphuric acid, 25 per cent solution	100 cc.

The preparation is then washed, dried and mounted. This method cannot be recommended as entirely reliable.

Staining for Differentiation of C. tuberculosis and C. smegma (Pappenheim's Method).

1. Fixed preparations are stained with steaming carbol-fuchsin in the usual way.

2. Pour off excess stain and without washing cover the film with the following solution pouring it off slowly and replenishing 4 or 5 times:

Rosalic acid	1 gram
Alcohol, absolute	100 cc
Methylene-blue	Added to saturation (about 0.65 gram)
Glycerin	20 cc.

3. Wash in water.

4. Dry and mount.

In films stained by the above method smegma bacilli which are acid-fast with the usual method are decolorized, whereas C. tuberculosis is not decolorized, appearing bright red in color.

Wright's Blood Stain. Wright's stain can be purchased from dealers in biological stains or it can be prepared in the laboratory, a number of workers preferring to produce their own Wright's stain. The stain is prepared in the following manner:

Make up a 1 per cent solution of methylene-blue (Grübler or make known to be suitable for polychroming) in a 0.5 per cent sodium bicarbonate solution. Place this in an Erlenmyer flask and subject it to flowing steam in the Arnold sterilizer for one hour. Allow this to cool and then add slowly sufficient of a 0.10 per cent aqueous solution of yellowish eosin to produce a purplish color with a metallic scum on the surface (about 5 parts of eosin to 1 part of methylene-blue). Close observation will show a fine, black precipitate. Filter the preparation discarding the filtrate. The precipitate is collected and dried and can be stored in powder form. For use 200 mgm. of the powder is dissolved in 120 cc. of pure methyl alcohol (acetone-free).

Wright's stain is used in the following way:

1. Cover the film preparation with undiluted stain and allow it to act for one minute. This results in fixation of the film by the methyl alcohol contained in the stain.

2. Add distilled water (or better, Na_2HPO_4 buffer solution of pH 6.8) dropping it on the slide until the stain mixture has a metallic surface luster. Let stain 2 minutes.

3. Wash off excess stain with distilled water allowing the last clear wash water to remain on film a few seconds to differentiate.

4. Dry and mount.

Giemsa's Stain

Azur-II eosin	1.5 grams
Azur-II	0.4 grams
Glycerin (c.p.)	125.0 cc.
Alcohol, methyl (c.p.)	125.0 cc.

Heat the glycerin and alcohol to 60°C. Dissolve the dyes in the warm glycerin and then add the alcohol. Allow the mixture to stand overnight at room temperature. It is then filtered and constitutes the stock solution. For use place 10 cc. of distilled water in a test tube, add 1 drop of a 1 per cent solution of potassium carbonate and 10 drops of the stock stain.

Films fixed 2 or 3 minutes with methyl alcohol are covered with this solution and stained for one-half to several hours. Over-staining, as a rule, does not occur even if the dye be permitted to act as long as 24 hours.

After subjecting the film to the stain for the desired length of time the slide is washed well in water, dried and mounted.

As a rule it is good practice to make sufficient of the dilution of Giemsa stain to nearly fill a Coplin jar. The slides can then be placed in the jar and stained for long periods of time without danger of drying.

Mann's Stain for Negri Bodies

1 per cent aqueous solution of methyl-blue	3.50 cc.
1 per cent aqueous solution of eosin	3.50 cc.
Distilled water	10.00 cc.

The methyl-blue and eosin solutions are made up and kept as separate stock solutions, only small amounts of the mixture being made at one time. A number of formulae for Mann's stain specify methylene-blue. The dye required is not methylene-blue but methyl-blue.

"Touch" preparations are stained 5 to 15 minutes, washed in water, dehydrated by passing rapidly through 50 per cent, 75 per cent, 95 per cent and absolute alcohol and cleared in xylol and cedar oil, equal parts. The Negri bodies stain brick-red, the nerve cells blue and the red blood cells an orange color.

Van Gieson's Stain for Negri Bodies

Distilled water	10 cc.
Saturated alcoholic solution rose-aniline-violet	2 drops
Saturated aqueous solution methylene-blue	1 drop

After fixing "touch" films in methyl alcohol flood slide with this stain and heat gently for one minute. Wash in water. The Negri bodies stain a pink or purplish red, the nerve cells blue. This stain should be mixed as needed.

Williams' Modification of Van Gieson's Method of Staining Negri Bodies.

Distilled water	30.0 cc.
Saturated alcoholic solution fuchsin	0.5 cc.
Saturated alcoholic solution methylene-blue	10.0 cc.

Allow "touch" preparations to partially dry in air and fix 10 seconds in neutral methyl alcohol containing 0.1 per cent picric acid. Drain off fixative and absorb excess with filter paper. Flood slide with stain and heat gently until it steams. Wash with water and blot dry. Negri bodies appear magenta with blue granules. Nerve cells stain blue and the red blood cells yellow or salmon color.

Mallory's Eosin-Methylene-Blue Method for Staining Bacteria in Tissue Sections. This method gives excellent results with tissue fixed in Zenker's fluid and embedded in paraffin.

1. Stain paraffin sections in 5 per cent aqueous solution of eosin for one hour in paraffin oven.
2. Wash in water.
3. Stain with the following for 15 minutes.

Methylene-blue	2 grams
Alcohol (95 per cent)	10 cc.
Distilled water	90 cc.

This is kept as a stock solution and when ready for use add 1 cc. of this solution to 9 cc. of a 1:1000 potassium carbonate solution.

4. Wash in water.
5. Differentiate and dehydrate in 95 per cent alcohol containing a few drops of a 10 per cent solution of colophonium in absolute alcohol. In carrying out this part of the procedure the section should be kept constantly in motion to insure uniform decolorization. The section must be examined several times with the microscope to determine when to discontinue the differentiation. As soon as the pink color has returned to the section and the nuclei are still blue, complete the dehydration rapidly in absolute alcohol.
6. Run through two changes of xylol.
7. Mount in balsam.

CAPSULE STAINS

Welch's Stain.

1. Make smear or film preparation in the usual manner and allow it to dry in the air.

2. Pour a little glacial acetic acid over the preparation and allow it to act a few seconds.

3. Drain off acid and without washing cover with aniline-gentian-violet solution (same as used in Gram's method). Pour this off and replace with more of the aniline-gentian-violet and repeat until all the acid is removed, leaving the last stain on 2 or 3 minutes. Wash with 2 per cent sodium chloride solution, mount by putting a drop or two of the NaCl solution on the preparation and drop a cover-glass on same.

Muir's Method.

1. Smear preparations are covered with Ziehl-Neelsen's carbol-fuchsin and gently heated for one-half minute, causing the stain to steam slightly.

2. Wash very lightly in 95 per cent alcohol and follow this immediately with a thorough washing in water.

3. Cover slide with the following mordant which is allowed to act 10 seconds:

Mordant

Saturated aqueous solution mercuric chloride	20 cc.
20 per cent aqueous solution tannic acid	20 cc.
Saturated aqueous solution potassium alum	50 cc.

4. Wash well in water.

5. Cover with 95 per cent alcohol for 1 minute.

6. Wash in water.

7. Stain with methylene-blue for $\frac{1}{2}$ minute.

8. Wash in water.

9. Dehydrate in alcohol, clear in xylol, mount.

Hiss's Copper Sulphate Method. In this method best results are obtained with cultures grown on serum media and in cases where the preparation is made direct from exudates, etc. If cultures not containing serum are to be used it is necessary to emulsify a little of the culture on the slide in a drop of beef or other serum and then smear this out, dry and fix by heat.

1. Cover the preparation with the following stain and heat gently for about $\frac{1}{2}$ minute allowing it to steam slightly:

Saturated alcoholic solution of fuchsin (or gentian violet)	5 cc.
Distilled water	95 cc.

2. Wash the stain off with a 20 per cent aqueous solution of copper sulphate.

3. Dry by blotting between filter paper. Do not wash.

The cell proper is a dark purple while the capsules appear as pale blue halos around the cell.

Huntoon's Method. Prepare a 3 per cent solution of sodium casinate (nutrose) in distilled water and heat it in the Arnold sterilizer for one hour. Remove and add 0.50 per cent phenol. Fill into tubes without filtering.

In making preparations for capsular staining a drop of nutrose solution is placed on a slide and a little of the culture (exudates, etc., cannot be used) is mixed into the same, streaked over the slide and allowed to dry. Do not heat fix. Stain for 30 seconds with the following preparation:

2 per cent aqueous solution phenol	100.0 cc.
1 per cent aqueous solution acetic acid	1.0 cc.
Lactic acid (concentrated)	0.5 cc.
Carbol fuchsin	1.0 cc.
Saturated alcohol solution basic fuchsin	1.0 cc.

Wash in water, dry.

India Ink Method. In this method a loopful of culture or material is simply mixed with a drop of India ink on a slide and the mixture then spread out in a film with another slide in the same manner a blood film is made.

Gram's Method. Gram's method of staining, carried out as outlined on page 87 frequently brings out capsules satisfactorily.

FLAGELLA STAINS

Staining flagella of bacteria is quite a difficult procedure. Unless all conditions are right, failure to obtain satisfactorily results is inevitable. First of all young cultures 15 to 18 hours old must be employed. Then the cover slips and slides must be scrupulously clean. In making the smear as little manipulation as possible must be employed to prevent breaking off the flagella. Drying must be rapid.

For the preparation of the smear or film for flagella staining proceed as follows: In a tube containing several cubic centimeters of water inoculate sufficient of a young agar culture to produce a slight turbidity of the water. Place in the incubator for $1\frac{1}{2}$ to 2 hours. Take a loopful of the fluid from the top and place it on a thoroughly clean slide gently spreading it out without making back and forth motions across the area. Dry rapidly. The preparation is then ready to be treated by one of the following methods:

Pittfield Method (Muir's Modification).

1. Cover film with the following mordant and steam very gently for one minute:

10 per cent aqueous solution tannic acid	10 cc.
Saturated aqueous solution mercuric chloride	5 cc.
Saturated aqueous solution alum	5 cc.
Carbol-fuchsin	5 cc.

This solution develops a precipitate which should be permitted to settle or it can be thrown down by centrifugalization. This preparation is unstable, keeping only about one week.

2. Wash well in water for two minutes.
3. Dry in incubator (do not blot).
4. Flood slide with following stain and steam gently for one minute:

Saturated aqueous solution of alum	10 cc.
Saturated alcoholic solution of gentian-violet or carbol-fuchsin	2 cc.

(This stain keeps only about 2 days.)

5. Wash well in water, dry and mount.

Van Ermengem's Method. Prepare film as outlined above, fixing gently by heat, taking care not to overheat as flagella will be burned off. Have on hand the following 3 solutions:

Solution 1

Osmic acid, 2 per cent solution	120 cc.
Tannic acid, 20 per cent solution	60 cc.
Glacial acetic acid	0.5 cc.

Solution 2

Silver nitrate, 0.50 per cent solution

Solution 3

Gallic acid	2.50 grams
Tannic acid	1.50 grams
Potassium acetate, fused	5.00 grams
Distilled water	175.00 cc.

1. Place the cover-slip or slide preparation in solution No. 1 and allow it to remain for one hour at room temperature. If desired the process can be hastened by boiling the preparation in solution No. 1 for 5 minutes.
2. Wash in water.
3. Next wash in absolute alcohol.
4. Immerse the preparation in the silver nitrate solution (solution No. 2) for 3 seconds.
5. Without washing place in solution No. 3 and move it about in this for a minute.
6. Place in the silver nitrate solution again and allow it to remain until it turns black.
7. Wash well in water and mount.

Zettnow's Method. Prepare the following mordant:

Tannin	5 grams
Distilled water	100 cc.
Heat to 60°C. and add	
5 per cent aqueous solution of tartar emetic	15 cc.

This mordant appears turbid on cooling but should clear up when the solution is heated.

Solution 2

Silver sulphate	0.50 gram
Distilled water	125.00 cc.

When ready for use add several drops of ethyl-amine (33 per cent solution) drop at a time, until a yellowish-brown precipitate forms and then dissolves.

In utilizing this method preparations (usually on cover-slips) are placed in a small evaporating dish containing a small quantity of the mordant and heated over a water bath for 6 or 7 minutes. The dish is removed from the water bath and when the solution has cooled so that a slight turbidity is noted the preparation is removed and washed well in water.

Hold the cover-slip preparation with a pair of forceps and pour on a few drops of the second solution and carefully subject it to gentle heating so that it steams very slightly and starts to appear black at the border of the preparation. Wash well in water, mount and examine.

METHODS OF STAINING SPORES

Abbott's Method. Preparations are made on cover-glasses or slides and heat fixed in the usual manner. Flood the preparation with Loeffler's alkaline-methylene-blue and heat over the flame until it commences to boil. Remove for a few seconds and heat again to boiling. Repeat this process several times avoiding continuous boiling.

2. Wash in water.

3. Decolorize with a 2 per cent alcoholic solution of nitric acid until no more blue comes out.

4. Wash in water.

5. Stain for a few seconds with a solution prepared by adding 10 cc. of a saturated alcoholic solution of eosin to 90 cc. of distilled water.

6. Rinse lightly in water, dry and mount.

By this method the spores are stained blue and the rest of the organism a pale pink.

Moeller's Method.

1. Fixed preparations are placed in chloroform and allowed to remain 2 minutes.
2. Wash in water.
3. Flood with a 5 per cent solution of chromic acid and warm until the solution just starts to steam. Allow acid to act about $\frac{1}{2}$ minute after steaming and then wash in water.
4. Flood with carbol-fuchsin and gently steam for 2 or 3 minutes.
5. Decolorize with a 5 per cent solution of sulphuric acid.
6. Wash in water.
7. Cover with methylene-blue solution for one minute.
8. Wash with water.

The spores are stained red, the body of the bacillus blue.

PART III

INFECTION AND IMMUNITY

CHAPTER VIII

Microörganisms and Disease; Resistance and Immunity

Hordes of bacteria and closely allied forms of lower life abound in nature. Fortunately, the vast majority of these microörganisms are not detrimental to the health of the higher animals. As a matter of fact a great many of such organisms serve a very useful purpose in the economy of things. Thus, the saprophytic bacteria, as a class, are important factors in splitting up dead animal and plant tissues, thereby producing from complex structures simple substances essential to the very existence of vegetable life. On the other hand, we find a relatively small group in the kingdom of microörganisms leading a parasitic life on man and the lower animals. Some of these parasitic forms cause no detectable disturbance in their host while others are responsible for various diseases. We thus recognize two important groups of organisms—*saprophytes* and *parasites.*

The saprophytes are found widely distributed in nature and normally attack only dead tissues of animals and plants. Occasionally, however, certain species of bacteria which ordinarily occur as saprophytes may become concerned in disease processes as secondary factors after some primary agent has properly paved the way. Further, the by-products of metabolism of a few of the saprophytic organisms are capable of causing disease in man and the lower animals. Thus, ptomaines and botulism toxin, produced by the activity of organisms outside of the body, are capable of causing serious disturbances in the health of man and animals ingesting same.

Of the parasitic organisms there are various species commonly found in different parts of the body (respiratory tract, mouth, intestinal tract, genital tract) which, so far as known, are harmless to the host. These are *non-pathogenic parasites*, i.e., while living a parasitic existence they do not produce disease in their host. On the other hand, there are certain parasitic organisms which, for a time, may exist as harmless parasites in the animal body but under certain favorable conditions may produce disease. Such microbes are *facultative pathogenic organisms*. Finally, there exists another group of microörganisms which are preëminently *pathogenic species* and when parasitic upon susceptible animals usually produce specific diseases.

When an organism succeeds in gaining entrance into the body of an animal and produces disease the phenomenon is designated an *infection.* When disease occurs as a result of activity of organisms which have previously

existed in the body without causing disturbances, the phenomenon is known as an *endogenous infection.* When disease results from organisms introduced from the outside we have an *exogenous infection.*

The degree of ability of an organism to produce infection is termed *virulence.* There is considerable variation in virulence between different species of microörganisms and also variation in virulence between different strains of a single species. With some bacteria infection may result from the introduction of a few or even a single organism into the body while with other species considerable numbers must gain entrance before infection takes place.

Some bacteria, under certain conditions, are often incapable, in themselves, of producing disease but may prove pathogenic when present with other organisms. Thus, the anaerobic tetanus organism, when present alone in a certain type of wound, may not be capable of producing disease because of the presence of free oxygen. If, however, a *mixed infection* exists and aerobic bacteria are present with the tetanus organism, in such symbiotic state Clostridium tetani may then propagate, produce its toxin and disease through the action of the aerobes in utilizing the free oxygen present. The well-known mixed infections seen in cases of fistulous withers, poll evil and lesions of the skin are other examples. Conversely, the presence of various bacteria are sometimes detrimental to the existence of other types. We have an example of this in the case of Pseudomonas pyocyaneous which when present with Clostridium chauvei is detrimental to the existence of the latter.

The *path* by which an organism gains entrance to the body is, in many instances, an important factor in determining whether or not infection will take place. For example, the organism of malignant edema and the virus of rabies may be ingested in large quantities and ordinarily will cause no disturbance. On the other hand, if they are introduced into the musculature through wounds disease is produced. Various anaerobic bacteria can be injected intravenously without causing disease whereas injections of the same into the musculature result in infection. A few organisms will infect only when the mode of entrance is by way of the alimentary tract. In the case of dourine in horses infection occurs by way of the genital tract. In another class of diseases an intermediate host is essential in spreading the infection.

In causing disease in man or the lower animals the inciting agent frequently shows special *affinity* for certain tissue. Thus, the Mycobacterium tuberculosis has a predilection for lung tissue, the rabies virus for nerve tissue, Alcaligines abortus for chorionic tissue, etc.

In order that the pathogenic microörganisms may produce infection and thus perpetuate their species, they possess, to a greater or lesser extent, structural characteristics and functional properties designed to aid them in accomplishing their mission. Thus, certain bacteria possess capsules, occur in spore form, produce toxins and various other types of by-products, all of which serve to promote the purpose of the invading organism. On the other hand, nature has endowed man and the lower animals with defensive mechanism to combat the attacks of these disease-producing forms of life. The unbroken skin is an effective barrier to most organisms. The mucous membranes, while permitting infection with certain microbes, forestall others, and, further, through their secretions mechanically rid themselves of large numbers of organisms. The saliva is unfavorable to the development of many microbes gaining entrance to the mouth, the gastric juices destroy numerous varieties of microörganisms and the bile exerts a destructive action on certain bacteria. Finally, if organisms succeed in passing these barriers and gain entrance to the deeper tissues they meet with a second line of defense in the form of phagocytes, various antibodies, etc., which will be discussed in more detail a little further along.

Thus, in the attempts of pathogenic microörganisms to produce disease and the efforts of the body to resist or overcome the invaders we have another example of the paramount problem of life—the struggle for existence. This struggle for supremacy between an infecting organism and the body constitutes an *infectious disease.* If the microbe wins death results. If the body is victorious recovery from the disease occurs.

When an infectious disease is readily communicated from animal to animal through direct or indirect contact it is also a *contagious disease.* All infectious diseases are not contagious. Tetanus, for example, is infectious but it is not spread from animal to animal through contact and is, therefore, not contagious. Thus, all contagious diseases are necessarily infectious but all infectious diseases are not necessarily contagious.

If a pathogenic organism succeeds in passing the agencies constituting the first line of defense and gains entrance into the body proper, whether or not infection takes place will depend, on one hand, upon the virulence of the invading organism, and, on the other hand, upon the *resistance* or *immunity* of the animal. The distinction between resistance and immunity is but slight. As a matter of fact, it is largely but one of degree. The normal body forces which are opposed to bacterial invasion are more effective in some individuals than in others and, further, they vary in the same individual under certain circumstances. Thus, we have conditions of *lowered resistance* or *increased susceptibility* when the general physical condition

of an animal is below par or when the animal is subjected to exposure, fatigue, starvation, etc. The term *immunity* designates a marked or unusual resistance of an animal to specific infection. In immunity we recognize two main types—*natural immunity* and *acquired immunity.*

Natural immunity, as the term implies, is congenital and may be *specific*, *racial* or *individual*. Thus, certain species of animals are naturally immune to disease to which other species are susceptible. The equine species, for example, is highly susceptible to glanders, while, on the other hand, the bovine species possesses a substantial natural immunity to the disease. The bovine species is very susceptible to aphthous fever (foot-and-mouth disease) whereas the equine species is naturally immune to same. Man is naturally immune to certain of the diseases of lower animals and the lower animals are resistant to certain of the diseases common to man. An oft cited example of racial immunity is that of the Algerian sheep which is highly resistant to anthrax while other sheep are very susceptible to such disease. Insofar as individual immunity goes, it is frequently observed that a particular individual in a given group may resist infection to which other individuals of the same species succumb. In many cases this immunity may be due to former infection of a mild type which caused no outward manifestations of disease, but in other cases it seems certain that the immunity is due to possession of greater individual resistance than that of most animals of the same species.

Acquired immunity is obtained either from active participation of the body in the process from which immunity results, in which case it is termed *active immunity*, or by a process in which the animal immunized plays no part in building up the "immune bodies," in which case we have what is termed *passive immunity*. Thus, if an animal develops an infectious disease, an attack of which confers immunity, and recovers from same, it is said to have an active immunity. Similarly, if a susceptible animal is artificially immunized, say against anthrax, by injecting it with anthrax vaccine, it has an active acquired immunity. On the other hand, if an animal is immunized against an infectious disease by injecting it with an immune serum, obtained from an animal which recovered from the particular disease or which was artificially immunized against same, such conferred immunity is spoken of as passive immunity. In such cases the "immune bodies" were already present in the serum injected as a result of activity on the part of the body of the animal furnishing the serum, the body of the recipient playing no part in the production of the protecting bodies. Active immunity is of greater duration than passive immunity, the latter ordinarily lasting but several weeks.

CHAPTER IX

Theories of Infection and Immunity; Phagocytosis; Opsonins and Opsonic Index; Antibodies

If the body defenses are incapable of preventing infection the organism produces disease. The exact mechanism by which organisms cause disease is not known in all cases. Some bacteria, such as Clostridium tetani, produce a powerful *exotoxin*, a so-called *soluble* or *true toxin* which is responsible for the symptoms of disease. Few organisms, however, produce exotoxins. On the other hand, a considerable number of organisms have been demonstrated to contain intracellular products which are toxic. Such toxic material is a constituent part of the cell and is only liberated when the cell is broken up. Such cell constituents have been designated *endotoxins*. When fluids containing the intact organisms are freed of same by filtration, the filtrate is non-toxic. On the other hand, the washed organisms may be shown to be toxic. A number of the pathogenic organisms undoubtedly function through such endotoxins.

The proof of the existence of a true or soluble toxin is found in ability to produce an *antitoxin* against same. In the case of endotoxins no true antitoxin can be produced. A more detailed discussion of true toxins and antitoxins will be found in the next few pages.

Aside from soluble toxins and endotoxins other products of bacteria metabolism undoubtedly play a part in disease production. Certain bacteria produce *hemolysin*, an agent capable of attacking the red blood cells and liberating their hemoglobin. Various streptococci and staphylococci are capable of producing a substance termed *leucocidin*, a product which tends to prevent or inhibit the phagocytic action of the white blood cells. Then, Bail and his students have maintained that bacteria are capable of secreting, in the animal tissues, a substance which has been termed *aggressin*. The function of this agent, as conceived by Bail, is to inhibit phagocytosis, thus permitting the organisms to multiply with less interference. As such agent exerts an aggressive action against the opponents of the invading bacteria Bail gave it the designation mentioned.

In support of his hypothesis, Bail showed that when exudates, collected from the region of lesions in fatal infections, are freed of bacteria and injected into a susceptible animal simultaneously with a culture analogous to that producing disease in the first animal but of much lower virulence,

the exudate would greatly enhance the virulence of the weak culture. The exudate was shown to produce no appreciable disturbance when injected alone. Bail further proved that animals could be immunized against infection with certain bacteria by injecting them with sterile exudates containing specific aggressins. Such immunity is explained on the ground that specific *anti-aggressins* are produced as a result of the reaction of the body against the aggressins introduced and that, subsequently, when the specific organism invades the animal body and commences to produce aggressins these are promptly neutralized by the anti-aggressins and the bacteria are then readily destroyed by the phagocytes.

Some authorities disagree with Bail in his conception of the nature of aggressins. They consider them as nothing more than endotoxins.

With the entrance of an invading organism into the body two principal agencies come into play. The first is a group of cells termed *phagocytes* which, under certain conditions, are capable of engulfing and destroying bacteria. The phagocytes are chiefly the leucocytes, particularly the polynuclear type, and the endothelial cells. The second agency consists of *ferments* or *antibodies* contained in the body fluids. These are capable of bringing about a destruction of the invading organisms and neutralizing their toxic products. These two agencies represent the ground structures upon which two well-known theories of immunity are based. Metchnikoff and his followers conceived *phagocytosis* as the principal factor in determining immunity. On the other hand, Nuttall, Fodor, Buchner, Flugge, Pfeiffer, Bordet, Ehrlich and various of their followers took the position that immunity depended, in the main, upon the *antibody* content of the body humors and that phagocytosis was merely a secondary process by which organisms already overcome by the antibodies were removed. Thus, the *phagocytosis theory* and the *antibody theory* of immunity arose. While neither theory is flaw-proof in its entirety, our present conception of immunity admits of a combination of parts of both theories. Thus, if we consider immunity as being due, in the main, to both phagocytosis and antibody activity, we are probably not far wrong.

While the leucocytes and endothelial cells of all individuals are capable of more or less phagocytic action when the body is invaded by microorganisms, the degree and success of phagocytosis is influenced by the degree of specific immunity. For example, if an animal susceptible to hemorrhagic septicemia is injected with a suspension of Pasteurella organisms the phagocytes will do their share in endeavoring to overcome the invasion, and some phagocytosis, even though very limited and of no avail, will occur. On the other hand, if an animal which has been immunized against hemor-

rhagic septicemia is injected a greater phagocytosis occurs. While an animal which is susceptible to a disease may not contain in its body fluids specific ferments or antibodies such as are possessed by an immune animal, nevertheless, it undoubtedly has, normally, ferments or substances which are antagonistic to invading bacteria even though incapable of successfully preventing infection.

To briefly summarize the defense forces of the body we find that first of all it is protected from the onslaughts of pathogenic bacteria by its physical coverings—the skin and mucous membranes. Second, that its various secretions play a part in disease prevention. The secretions of the mucous membrane mechanically remove large numbers of bacteria; the gastric juices and bile are bactericidal. Then we find that if a microörganism succeeds in penetrating the outside barriers and gains entrance to the body proper it meets with the offenses of the phagocytes and various ferments or antibodies.

PHAGOCYTOSIS

The term phagocyte is derived from the Greek roots "phagein"—to eat, and "kytos"—cell. Thus, the rôle of the cell which serves as a phagocyte is to eat or engulf and destroy invading organisms.

Hayem,[1] in 1870, was probably the first investigator to record the observation of the cells we now know as phagocytes engulfing bacteria. This investigator, however, did not suspect the nature of the phenomenon but was inclined to believe that this was one way in which bacteria were transported to different parts of the body. In 1874 Panum[2] vaguely indicated that the presence of bacteria within leucocytes might possibly be interpreted as evidence of antagonistic action on the part of such cells against the invading microbes and part of the body's mechanism in overcoming infection. In 1883 Metchnikoff,[3] who had taken up the idea of phagocytosis, presented his first report on the subject. As a result of this and subsequent work Metchnikoff propounded the theory that an immune animal owes its immunity to the ability of its phagocytes to readily and effectively engulf and destroy invading organisms.

Metchnikoff recognized two types of phagocytes—*motile* or *wandering* and *fixed*. The leucocytes constitute the motile type while the endothelial cells and certain connective tissue cells of the spleen and lymph nodes are considered as fixed types. Further, Metchnikoff designated the polymor-

[1] Hayem, Comp. Rend. d. l. Soc. Biol., 1870.
[2] Panum, Virchow's Archiv., vol. ix, 1874.
[3] Metchnikoff, Arbeit. a. d. Zool. Inst., Wien, vol. v, 1883.

phonuclear leucocytes *microphages* and the large mononuclear leucocytes and the fixed phagocytes *macrophages*. The microphages are considered most active.

The mechanism of phagocytosis in an infection is somewhat as follows: When an infection occurs the presence of the bacteria serves to attract the leucocytes, a phenomenon spoken of as *positive chemotaxis*. The leucocytes pass through the walls of the regional capillaries into the invaded tissue area. At the same time a serous exudate collects and we have swelling, pain, heat and redness, the cardinal symptoms of inflammation. The leucocytes, chiefly polymorphonuclears, which have migrated to the invaded area, together with such other phagocytic cells as may be locally present, engulf and destroy the bacteria through a sort of digestive action. If in the process numerous phagocytes are destroyed we have an accumulation of *pus* which consists largely of dead leucocytes, bacteria and tissue detritus in inflammatory exudate.

Metchnikoff observed that phagocytes functioned more actively when present in immune serum than they did in normal serum and accounted for such greater activity by assuming the presence of substances in the immune serum capable of exerting a stimulating effect on the phagocytes. Several other investigators suggested that, rather than being due to a stimulating action on the phagocytes, the difference might be the result of substances in the immune serum which lowered the resistance of the bacteria thus rendering them more susceptible to phagocytosis. Finally, in 1903, Wright and Douglas,[4] utilizing the principle of a method devised by Leishman[5] the year before (1902) for the observation of phagocytosis *in vitro*, definitely demonstrated that blood serum contains a substance which serves to prepare the bacteria for attraction and engulfment by the phagocytes. They termed this serum constituent *opsonin*. While present to a certain extent in normal serum the opsonin content of immune serum was found to be far greater. Further, it has been shown that the normal opsonin is less stable on standing or when exposed to heat than specific opsonins in immune serum.

Following the demonstration of opsonin as a factor in phagocytosis, Wright undertook the development of a satisfactory method for determining the opsonic activity of the serum of immune individuals and particularly of individuals being treated with vaccines or bacterins. It was Wright's idea that in vaccine therapy there occurs, shortly after the injection of the

[4] Wright and Douglas, Proceedings Roy. Soc., 1903, 72.
[5] Leishman, British Med. Jour., vol. i, 1902.

various doses of vaccine, a *negative phase* or period when the individual's protective elements are temporarily diminished. In order to avoid administering a dose of vaccine to a patient during this negative phase Wright concluded that the opsonic action of the serum would serve to indicate whether or not it was advisable to give an injection of vaccine at a particular time. The outcome of this work was the development of a method for determining what Wright designated the *opsonic index* of an individual's blood serum. This index is arrived at by comparing the opsonic action of the patient's serum with that of a normal serum.

Before going into the details of the opsonic index it is desired to point out that Leishman ahead of Wright actually conducted tests which, in a general way, were really measurements of opsonic activity. Leischman, however, looked upon his results as demonstrations of stimulated phagocytic activity rather than opsonic action as we now know it.

Opsonic Index—Wright's Method. For the determination of the opsonic index we must have at hand the individual's serum, normal serum (preferably the pooled serum of three or four individuals), a suspension of fresh leucocytes and a suspension of the particular bacteria. In dealing with the lower animals the specimen of blood is taken by means of veni-puncture and allowed to clot. The separation of the serum can then be accomplished by centrifugalizing the blood for 10 or 15 minutes. After such procedure the clear serum can be pipetted or carefully poured off of the clot into a sterile tube.

The leucocyte suspension is obtained by the following method: Several cubic centimeters of blood, preferably from the same species of animal whose opsonic index is to be determined, are collected in an equal volume of citrate solution (1.5 per cent sodium citrate in physiological salt solution). This mixture is centrifugalized for 5 or 10 minutes. It will then be found that the cells have packed at the bottom with the leucocytes (of less specific gravity than the red cells) forming a creamy upper stratum. The supernatent fluid is carefully removed and with equal care the superficial layer of leucocytes collected by means of a capillary pipette and placed in another tube containing a little physiological saline solution for washing the cells. The process of centrifugalization is repeated, the supernatent saline solution drawn off and the final suspension of leucocytes made in a small quantity of saline solution.

The bacterial suspension is prepared by placing several loopfuls of a young culture (18 to 24 hours old) of the particular organism in a little physiological saline solution in a small test tube. The same should be stoppered and the mixture vigorously shaken to insure an even suspension of

the organisms. For use a small amount of such suspension is poured into a clean watch glass.

With the reagents thus prepared we proceed with the actual test. Several capillary pipettes, equipped with rubber nipples, are marked with a wax pencil about 2 to 3 cm. from the end. Into one of these pipettes draw up some of the leucocyte suspension until it just reaches the pencil mark. Admit a small air bubble and then fill the pipette up to the mark with the suspension of bacteria. Admit another air bubble and draw up some of the serum to the mark. We now have in the capillary portion of the pipette approximately equal quantities of the leucocyte suspension, bacterial suspension and serum, separated by small air bubbles (Fig. 18). By slight pressure on the rubber nipple force all of the material in the pipette out on a watch glass and thoroughly mix it by successively drawing it up in the pipette and forcing it back on the watch glass, avoiding bubbling. Finally draw all of the mixture that can be collected into the pipette taking precautions to have it well up from the capillary end which is now sealed in a flame. This entire process is repeated with the normal serum and the sealed pipettes

Fig. 18. Wright Pipette for Opsonic Index Determination

are then incubated at 37.5°C. for 15 to 30 minutes. Following incubation the sealed end of the pipette is broken off, the material again mixed, and a drop or two placed on a slide and spread by means of another slide in the same way a blood film is prepared. The preparation is then stained with Wright's or some other satisfactory blood and bacteria stain. The same procedure is, of course, followed with both the questionable and normal serum. The next step is to examine the preparations with the oil immersion lens and count the bacteria found in 100 leucocytes and average same. The average number of bacteria contained in one leucocyte gives the *phagocytic index*. By dividing the phagocytic index of the serum under test by the phagocytic index of normal serum we obtain the *opsonic index*. For example, we find the average number of bacteria in the leucocytes treated with an immune serum to be 8 whereas with a normal serum the average is 4. Thus we have $\frac{8}{4}$ = opsonic index of 2.00.

There have been several additional methods advocated for the determination of the opsonic activity of serum but at the present time neither Wright's nor any other method is practiced to any appreciable extent. The range

of error is such that for ordinary purposes the test is hardly practicable. The test has, however, served to bring out several important points in connection with phagocytosis and opsonins but today it is of more historical than practical interest.

THE ANTIBODY OR HUMORAL THEORY OF IMMUNITY

Of the phagocytosis theory of immunity and the antibody theory the latter is the more far-reaching and explains, in a fairly satisfactory manner, a number of the manifestations of immunity which, obviously, cannot be explained by the first theory.

The humoral theory of immunity orginated with Buchner[6] after he had succeeded in confirming the finding of Nuttall[7] that normal blood serum contained substances detrimental to bacteria. Buchner designated this unknown bactericidal constituent *alexin* (protective material). This agent, as Nuttall has pointed out, is most apparent in fresh blood serum and is thermolabile, being destroyed by a temperature of 55°C. Buchner considered alexin a general bactericidal agent equally active against all varieties of organisms.

In 1890 Behring and Kitasato[8] reported that animals could be actively immunized against diphtheria and tetanus toxins and that the sera of such immunized animals would protect susceptible animals against doses of toxin which would ordinarily prove fatal. Behring designated the protective substances in such immune serum *antitoxin*. Following this work of Behring and his associates, it was found that antitoxins could be prepared for various other toxic substances including certain poisons other than those produced by bacteria. This work threw considerable light on immunity in diseases in which a true toxin was concerned.

Pfeiffer,[9] in a brilliant piece of work, presented about five years after Buchner's report on alexin, showed that the bactericidal constituent in the blood sera of guinea pigs immune to cholera was *specific* for cholera organisms. He further demonstrated that *in vitro* the bacteriolytic action of the immune serum was lost if the serum was heated, but when heated and injected into the *body of an animal* it was effective to the same extent as the unheated serum. Bordet[10] confirmed Pfeiffer's findings and showed that when immune serum, heated to 50° to 60°C., was added to cholera organisms

[6] Buchner, Centralbl. f. Bakt., 1889.
[7] Nuttall, Zeit. f. Hyg., i, 1886.
[8] Behring and Kitasato, Deut. Med. Woch., no. 49, 1890.
[9] Pfeiffer, Zeit. f. Hyg., xviii, 1894.
[10] Bordet, Ann. de l'Inst. Past., vol. 12, 1898.

in a test tube the bacteriolytic property of the serum was not then manifested but that it could be brought into action by the addition of a little fresh, normal serum. Thus, it became apparent that the specific bacteriolytic substance present in the immune serum would act only in the presence of some thermolabile agent which was a constituent of both immune and normal serums. The bacteriolytic substance became known as "*bacteriolysin*" and the thermolabile, activating agent was recognized as Buchner's *alexin* or, as Ehrlich later termed it, *complement* (necessary to complete a reaction).

The demonstration of various other types of antibodies followed rather rapidly. In 1896 Gruber and Durham[11] reported the observation of agglutination or clumping of bacteria when the same were treated with specific immune serum. They designated the antibodies responsible for the phenomenon *agglutinins*. The following year (1897), Kraus[12] reported that certain culture filtrates, when brought in contact with specific immune sera, would result in the formation of precipitates. He termed the antibodies in the immune serum responsible for this reaction *precipitins*. Then, in 1898 came the discovery of Bordet[13] that red blood cells of one species injected into an animal of another species gave rise to the production of antibodies capable of causing a hemolysis of the foreign cells. This hemolytic antibody has been termed *hemolysin*.

Substances (bacteria, toxins, cells, etc.) which, when introduced into the body of an animal, stimulate the production of antibodies are termed *antigens*.

With the discovery of various types of antibodies considerable speculation developed as to the manner in which they were produced and the way they functioned. Ehrlich,[14] in studying the effect of antitoxin on its specific toxin, arrived at the conclusion that the reaction was one of neutralization of the toxin by the antitoxin in much the same manner an acid is neutralized by a base. Up until this time it was considered that antitoxin functioned through ability to attack and destroy toxin. Ehrlich's conception of antitoxin-toxin neutralization was soon accepted by various other investigators.

Next followed Ehrlich's detailed explanation of the mechanism of antitoxin production and toxin neutralization by antitoxin. This was incorporated in his now well-known *side-chain theory of immunity*. This theory was extended to explain the method of production and functioning of other

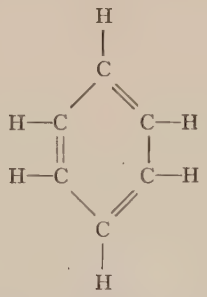
[11] Gruber and Durham, Munch. med. Woch., 1896.
[12] Kraus, Wien. klin. Woch., xxxii, 1897.
[13] Bordet, Ann. de l'Instit. Pasteur, 1898.
[14] Ehrlich, Deut. Med. Woch., 1891.

types of antibodies other than antitoxins. Thus, Ehrlich placed antibodies in three Orders. The *First Order* included the *antitoxins*, the *Second Order* takes in *agglutinins* and *precipitins*, while the *Third Order* deals with *cytolytic antibodies*, their production and method of functioning.

It is desired to emphasize the fact that Ehrlich's side-chain theory cannot, by no means, be considered as having definitely settled the exact or precise means by which the various antibodies are produced and the way they function. As a matter of fact, evidence of a definite nature has been brought forth to show that some of Ehrlich's conceptions, in part at least, are erroneous. To go into a discussion of all such work would necessitate a full discussion of immunity from all angles which is not the purpose of this book. It must be understood that immunity is, in itself, a broad subject which to treat of, at all properly, would require an entire volume. Ehrlich's side chain theory has, however, sufficed to explain, in a fairly tenable way at least, many problems of immunity and has played a tremendous part in developing the subject. Further, it has served as a practical working basis for much of our investigation and activity in the field of immunology.

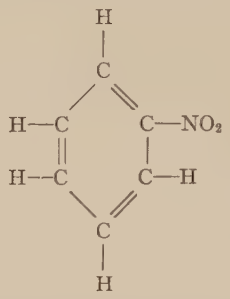
TOXINS AND ANTITOXINS (HAPTINES OF EHRLICH'S FIRST ORDER)

In chemistry a well-known hypothesis regarding the structure of organic compounds of the benzol group is that propounded by Kekule in which the carbon atoms are pictured as being arranged in a ring. Taking benzene with its formula C_6H_6, as an example, we construct the so-called "benzene ring." The six carbon atoms form a ring in which each atom is connected with the neighboring atom on one side by two affinities and to the neighboring atom on the other side by one affinity. This leaves each carbon atom with one affinity with which it connects the hydrogen. This can be illustrated as follows:



It is possible to replace each of these hydrogen atoms with some other group. For example, one atom might be replaced by the molecular group

NO_2. This would give the chemical formula $C_6H_5NO_2$ (Nitrobenzene) and can be diagramatically pictured as follows:



In the chemical hypothesis the NO_2 group, attached to one of the carbon atoms is designated a *side-chain*. Through this side-chain other substances can be brought into relation with the main structure.

Ehrlich, in formulating his side-chain theory of immunity, pictured body cells as having a variety of side-chains or *receptors* capable of uniting with different substances both of a nutritional or beneficial character and of a toxic or detrimental nature. It was considered that toxin could only prove detrimental to a cell by being bound to it through the agency of side-chains or receptors. The receptors which happen to be suitable for binding the toxin to the cell are rendered incapable of performing their normal physiological function when engaged with the toxin. Consequently, the cell, if it is not vitally injured, to compensate for this loss produces more receptors of the same character and, as Weigert hypothesized, the reproduction is in excess of what is actually needed to replace the receptors lost through combination with the toxin. The excess side-chains or receptors are shed by the cells, gain entrance to the circulation and constitute *antitoxin*. Then, if the animal is subsequently infected, the toxin produced is bound by these free, specific receptors (antitoxin) and thus rendered inert before it has an opportunity to combine with the receptors of the cells. It follows, of course, that the type of receptor which will bind or unite one type of toxin molecule to the cell differs for each toxin, otherwise immunization against one type of toxin would afford protection against others.

The *toxin molecule* has been shown to consist of two groups of elements One group serves to unite the toxin to the cell receptors and is thermostable. This is known as the *haptophore group*. The other portion is thermolabile and is the constituent which produces the toxic effect. This group is designated the *toxophore group*. These two groups can be demonstrated by heating a definite amount of toxin at 58°C. for one-half hour and adding to it

an amount of antitoxin just sufficient to neutralize an amount of unheated toxin equal to that heated. If the mixture is then incubated for one hour at room temperature and an amount of unheated toxin equal to the amount of heated toxin is added and incubated for another hour, animal inoculation tests will show that the mixture is toxic. In other words, the heated toxin, while incapable of producing injury, is, nevertheless, through its haptophore portion, able to neutralize antitoxin. A toxin element which has had its toxaphore group destroyed is termed a *toxoid*. Additional evidence of the ability of toxoids to engage cell receptors is found in the observation that injections of toxoids stimulate the production of antitoxin.

For the details of preparing and standardizing antitoxin the reader is referred to the section on the Preparation of Biological Products.

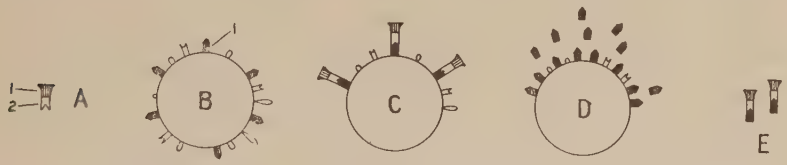


FIG. 19. DIAGRAMMATIC REPRESENTATION OF ANTITOXIN PRODUCTION ACCORDING TO EHRLICH'S SIDE-CHAIN THEORY

A, toxin molecule; *1*, haptophore group; *2*, toxophore group. *B*, body cell with various types of receptors; *1*, receptor suitable for binding toxin. *C*, toxin in contact with cell through receptor. *D*, over-production of the particular receptor, excess of same being thrown off by cell as antitoxin. *E*, toxin neutralized by antitoxin.

AGGLUTININS AND PRECIPITINS (HAPTINES OF EHRLICH'S SECOND ORDER)

Ehrlich's *second order* of antibodies includes agglutinins and precipitins. The mechanism concerned in the production of agglutinins, as conceived by Ehrlich, is as follows: When an animal becomes infected or is injected with an antigen capable of stimulating the production of agglutinins, a substance which has been termed *agglutinogen* is produced or liberated by the invading organism, or is present in the antigen injected. This agglutinogen is either contained in the bacteria and liberated when disintegration occurs, or it is a biproduct of bacterial metabolism. The agglutinogen finds a type of body cell receptor with which it can combine and, as in the case of toxin union with the cell receptors, the agglutinogens engaging suitable receptors render the latter incapable of performing their regular duties for the cell. This stimulates the cell to an overproduction of this particular type of receptor, the excess of which are shed by the cells and liberated in the blood serum as *agglutinins*. The *agglutinin* is conceived as being more complex than the

antitoxin. It possesses a *haptophore portion* which serves to unite it to the bacterial cell and a *zmyophore group* by means of which it causes clumping or agglutination. The haptophore group is thermostable whereas the zymophore group is thermolabile, being destroyed by a temperature of 65° to 75°C. An agglutinin which has had its zymophore group destroyed is designated an *agglutinoid*.

Precipitins are, according to Ehrlich's views, produced in much the same way as agglutinins. The stimulating agent, consisting of a haptophore group, is known as *precipitogen*. This attaches to a suitable cell receptor and an overproduction of such receptors are produced and liberated as *precipitins*. Like the agglutinins, the precipitins are composed of a thermostable *haptophore group* and a thermolabile *zymophore group*, the latter

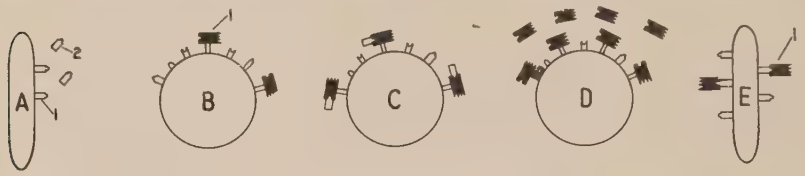


FIG. 20. DIAGRAMMATIC REPRESENTATION OF AGGLUTININ PRODUCTION ACCORDING TO EHRLICH'S SIDE-CHAIN THEORY

A, bacterial cell with attached (*1*) and free (*2*) agglutinogens. *B*, body cell with receptor (*1*) with which the agglutinogen is capable of combining. *C*, agglutinogen in contact with cell through suitable receptor. *D*, over-production and shedding of such receptors as agglutinins. *E*, agglutinin united to agglutinogen receptor of bacterial cell.

according to the theory, being responsible for the antibody's precipitating ability.

Our knowledge of antibodies of this Order and their functions has been put to practical use in the development of tests for the diagnosis of infections and the identification of various organisms. Thus, our agglutination, agglutinin absorption, and precipitin tests which are so extensively used for a variety of purposes have been developed.

For details of the agglutination and precipitin tests of interest in veterinary medicine see the section on Serology.

BACTERIOLYSINS AND HEMOLYSINS (HAPTINES OF EHRLICH'S THIRD ORDER)

As has been previously stated, the first observation of *lytic* or dissolving substances in immune serum was noted by Pfeiffer in his work with the cholera vibrio. Then, Bordet, and at about the same time, Belfanti and

Carbone,[15] discovered that animals injected with blood cells of another species produced lytic antibodies against the foreign cells.

The *cytolytic* antibodies destroying bacterial cells are designated *bacteriolysins*, while those acting upon red blood cells, liberating their hemoglobin, are termed *hemolysins*. Where a dissolution of bacterial cells has been brought about the process is spoken of as *bacteriolysis*. Where the

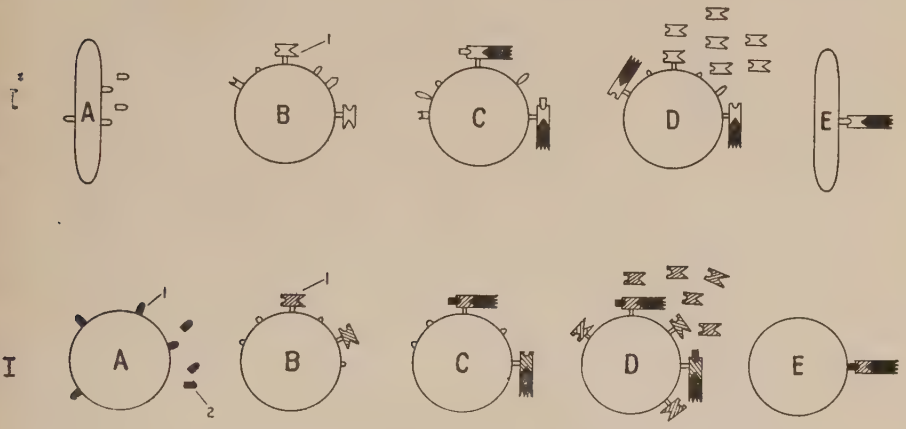


FIG. 21. DIAGRAMMATIC REPRESENTATION OF THE PRODUCTION OF CYTOLYTIC ANTIBODIES ACCORDING TO EHRLICH'S SIDE-CHAIN THEORY

I. *A*, bacterial cell with attached and liberated antigenic receptors. *B*, body cell with various receptors including a type (*1*) suitable for the attachment of the antigenic receptor. *C*, antigen united with cell through proper receptor; complement (the black figure) is also united to the cell through the complementophilous portion of the same receptor. *D*, over-production of such receptors and liberation of same as bacteriolytic amboceptors. *E*, such amboceptor attached to bacterial cell through its antigenic receptor. Activated by the complement the amboceptor produces bacteriolysis.

II. *A*, red blood cell with attached and liberated antigenic receptors. *B*, body cell with suitable receptors (*1*) for the antigen. *C*, antigen and complement attached to receptor. *D*, over-production of receptors and liberation as hemolytic amboceptors. *E*, hemolytic amboceptor attached to red blood cell and activated by complement produces hemolysis.

hemoglobin of red blood cells has been liberated *hemolysis* is said to have occurred. Ehrlich grouped these cytolytic antibodies in his *Third Order* and explained their production and method of functioning as follows:

When bacteria or foreign blood cells are injected into an animal certain of the antigenic side-chains or receptors, which they are conceived as having, are thrown off and unite with suitable receptors of the body cells. The

[15] Belfanti and Carbone, Giornala della R. Acad. di Torino, July, 1898.

particular type of body cell receptor with which they unite possesses two *haptophore groups*, one, a *cytophilous type*, and the other, a *complementophilous type* which serves to bind alexin or complement. Complement is normally present in the blood and enables this type of receptor to carry out the functions for which it exists. The union of the antigenic receptor with this cytophilous portion of the body cell receptor prevents this receptor from carrying out its normal function and as a result the cell is stimulated to an overproduction of this type of double haptophore receptor, the excess of which is thrown off from the cells and enters the circulation. This type of antibody is termed an *amboceptor*. When they result from the stimulus of bacteria or bacterial products they are termed *bacteriolytic amboceptors*. When foreign red blood cells serve as the stimulus the antibody is designated *hemolytic amboceptor*.

When bacteriolytic amboceptor comes in contact with the specific bacteria which stimulated its production it unites with same by its haptophilous portion and, in the presence of complement, by its complementophilous group to the complement, causing a disintegration of the bacteria—bacteriolysis. When hemolytic amboceptor, under similar conditions, comes in contact with red blood cells such as served as a stimulus for its production, it unites with the cell through its haptophilous group and with the complement by its complementophilous portion and brings about hemolysis.

Other types of cells, aside from bacteria and blood cells have been shown to be capable, to a limited extent, of stimulating the production of cytolytic antibodies when introduced into the bodies of experimental animals. The work along this line, however, has been comparatively limited and has not gained particular importance.

The antibodies of this order (bacteriolysins, hemolysins) together with complement, and the theory by which they unite and function, serve as a basis for our well-known *complement-fixation tests* which have proved of inestimable value in the diagnosis of certain infectious diseases. For a more detailed discussion of such tests the reader is referred to the section on Serology.

CHAPTER X

Hypersensitiveness (Allergy—Anaphylaxis)

The term "hypersensitiveness," as here used, designates that group of conditions in which certain individuals manifest a marked susceptibility to various agents, particularly foreign proteins, which ordinarily are harmless for normal individuals. The subject is commonly discussed under such headings as "*anaphylaxis*," "*allergy*," "*serum sickness*," "*food idiosyncrasies*," "*drug idiosyncrasies*," "*pollen hypersensitiveness*," etc.

At the present time there is considerable disagreement among investigators as regards the nature of some of these manifestations of hypersensitiveness. Further, there is a great deal of confusion in the use of terms which have been associated with the subject. For example, some authorities consider all of the various types of hypersensitiveness as but different manifestations of anaphylaxis. These same investigators use the term "allergy" to designate one group of anaphylactic reactions. On the other hand, other authorities employ "allergy" as the general term to cover the entire group of manifestations of hypersensitiveness and recognize anaphylaxis as one type of allergic reaction. In this work *allergy* is recognized as a broad term covering *any altered degree of reactivity*. It could thus be properly used to designate a condition in which an animal's usual reactivity to a certain stimulus was either *increased* or *decreased*. However, under the subject of "Hypersensitiveness" we, obviously, deal only with manifestations of increased susceptibility, thus allergy, as here used, is analogous with hypersensitiveness and covers the entire group of reactions of hypersensitiveness. We restrict the use of the term *anaphylaxis* to those reactions of hypersensitiveness which result from true antigen-antibody activity as hereinafter described.

The term *anaphylaxis* (meaning without protection) was first employed by Richet.[1] This investigator found that when a dog was injected with a toxic substance obtained from mussels known as "mytilocongestin," in an amount insufficient to cause symptoms of poisoning, a second injection on a subsequent date, of even a smaller amount, caused profound illness and in some cases death. This clearly indicated that the first injection had rendered the animal more sensitive to the preparation. Consequently

[1] Richet, Ann. de l'Instit. Pasteur, 1907–08.

Richet designated the condition as "*anaphylaxis*" as being opposed to *prophylaxis*.

Another observation of importance in this field was made by Arthus.[2] He injected rabbits subcutaneously with horse serum, repeating the process a number of times after intervals of six days. Following the first two or three injections the serum was promptly absorbed with but little evidence of local reaction. Subsequently, however, the injections were followed by pronounced local edema and towards the last resulted in the skin over the site of injection becoming gangrenous. When such animals were given serum intravenously they developed marked symptoms of a serious nature and in a number of instances died. This phenomenon has been designated the *phenomenon of Arthus*. It can be demonstrated with various antigens other than horse serum.

In 1904 Theobald Smith[3] observed that guinea pigs which had previously received injections of diphtheria toxin-antitoxin mixture, would, if injected ten days or more subsequently with a small amount of normal horse serum, develop a profound reaction and in a number of instances die. The horse serum in the form of antitoxin in the mixture first administered had sensitized the animal to normal horse serum so that the second injection of same resulted in anaphylactic shock.

Then came the work of Rosenau and Anderson,[4] and various others, in which it was shown that hypersensitiveness to certain proteins was specific for same; that a definite period of incubation must elapse between the sensitizing dose and the anaphylactic dose; that the hypersensitivity was transmissible from a mother to its young and that an animal could be rendered passively susceptible to anaphylactic shock by injecting it with the blood of a sensitized animal. It was also shown that animals could be rendered sensitive to other foreign proteins such as egg albumin, peptone, casein, etc.

In so far as our present knowledge goes, there is no evidence to indicate that anaphylaxis can be produced by any substance other than a protein.

There is a marked variation in susceptibility of different animals to anaphylactic reactions. The guinea pig is well-known for its susceptibility in this respect and is usually the species selected for demonstrating the phenomenon. Such animal can be sensitized with a very small amount of antigen (a minute quantity of horse serum, egg white, etc.) and after about

[2] Arthus, Compt. rend. de la Soc. Biolog., 55, 1903.

[3] Smith, Theobald, Jour. Med. Res., 1904.

[4] Rosenau and Anderson, U. S. Hygienic Lab. Bulletins Nos. 29 (1906); 30 (1906); 36 (1907); 45 (1908); Jour. Med. Res., xv, 1906, xvi, 1907; Jour. Inf. Dis., iv, 1907.

15 days if injected with the same type of protein it will develop, usually within a few minutes to one-half hour, symptoms of uneasiness (pawing of the nose, twitching, etc.), disturbed respiration, subnormal temperature, coughing and convulsions. In many instances the animal will die, death being due to asphyxia following spasm of the musculature of the bronchioles, the latter condition characterizing the phenomenon in guinea pigs. The lungs of guinea pigs dead from anaphylactic shock are markedly distended due to contraction of the smooth muscles of the alveoli which results in retention of the inspired air. While a minute amount of antigen will frequently suffice, in administering the anaphylactic dose of protein for the demonstration of anaphylactic reactions it is well to give a larger amount than that used for sensitization.

Rabbits are not as easily sensitized as guinea pigs. It usually requires several injections of antigen before they are rendered susceptible. In these animals the reaction is not characterized by bronchial spasm but by a circulatory disturbance due to acute dilatation of the right heart and marked contraction of the pulmonary vessels. This latter condition is due to spasm of the muscular coat of the lung arterioles.

In dogs anaphylactic shock brings about a marked engorgement of the liver and congestion throughout the entire splanchnic circulation.

It is to be noted that while the principal seat of anaphylactic reactions varies in the animals mentioned, it appears to coincide with the distribution of smooth muscle tissue, the bronchi in guinea pigs, the pulmonary arterioles in rabbits and the hepatic vessels in the dog, all having unusually well-developed, smooth musculature.

There have been a number of theories advanced to explain anaphylactic reactions. One which seemed very plausible and which appealed to many was that sponsored by Vaughan and Wheeler[5] and a number of other investigators. Vaughan and Wheeler explained the phenomenon as follows: The first injection of foreign protein stimulates the production of antibodies capable of splitting up such protein. When a second injection of the particular protein is administered it is rapidly split up by the specific antibodies and in the process toxic split-products quickly "swamp" the system causing an acute intoxication which is recognized as an anaphylactic shock.

Such explanation would appear entirely logical. In the first place the production of a toxic substance (*anaphylatoxin*) through protein cleavage has been amply demonstrated. Then, it is a well-known fact that the body,

[5] Vaughan and Wheeler, Jour. Inf. Dis., iv, 1917.

when properly stimulated, does produce certain types of *lytic* antibodies. Thus, the hypothesis which at once suggests itself is that the first injection of foreign protein stimulates the gradual production of an abundance of specific proteolytic antibodies which, in the presence of complement, cause a rapid disintegration of the particular protein when subsequently administered, thereby suddenly liberating sufficient anaphylactoxin to produce the reaction we recognize as anaphylaxis.

In spite of the fact that this explanation of anaphylaxis is quite logical, subsequent investigations indicate, rather definitely, that it is erroneous. The findings of Schultz,[6] Dale[7] and Weil[8] are particularly opposed to the protein cleavage hypothesis. These investigators demonstrated that when a protein is brought in contact, under proper conditions, with a washed segment of the uterus of a virgin guinea pig which has been sensitized to the particular protein, marked contraction of the muscle occurs. The contractions of the uterus segment start *immediately* following the addition of the protein to the bath in which the segment is suspended, the period of time involved being far less than would be required for protein cleavage. This finding also indicates that complement is not necessary in the reaction.

Additional evidence that anaphylactic shock does not occur as a result of an antigen-antibody reaction in the blood is found in investigations which have shown that in rendering an animal passively susceptible to such type of shock a period of at least four or five hours must elapse between the administration of the blood from a sensitive animal and the inoculation of the particular protein.

That anaphylaxis is not the result of the rapid splitting up of protein by antibodies in the blood, is further suggested by the observations of several investigators that the presence of an abundance of free antibodies in the circulation actually tends to prevent an anaphylactic shock by combining with the antigen before it has the opportunity to connect with the cells.

Leaving aside a lengthy discussion of the various explanations of anaphylaxis which now appear untenable, we find ourselves in accord with the views of Wells, Zinsser and others who believe that *real anaphylaxis results primarily from an antibody-antigen reaction which takes place upon or within the cell.*

[6] Schultz, Jour. Pharm. and Exper. Therap., i, 1910.
[7] Dale, Jour. Pharm. and Exper. Therap., iv, 1913.
[8] Weil, Jour. Med. Res., 1913, 27; 30, 1914.

Wells[9] has nicely summed up the criteria which he believes must be met before a condition can be regarded as one of true anaphylaxis. They follow:

1. The observed toxicity of the injected material must depend upon the sensitization of the animal; i.e., the substance must not produce similar symptoms in non-sensitized animals.

2. The symptoms produced must be those characteristic of anaphylactic intoxication as observed in the usual reactions with typical soluble proteins, being therefore the same for all antigens with the same test animal, but differing characteristically with each species of animal.

3. It should be possible to demonstrate passive sensitization with the serum of sensitized animals.

4. It should be possible to demonstrate typical reactions in the virgin guinea pig uterus strip.

5. It should be possible to demonstrate amelioration or prevention of the bronchial spasm in guinea pigs by proper use of atropin and epinephrin.

6. The possibility that the observed symptoms are caused by capillary thrombosis or embolism must be excluded.

7. After recovery from anaphylactic shock there should be exhibited a condition of desensitization under proper conditions.

Drug Idiocyncrasies. Hypersensitiveness of certain individuals to various drugs is a rather common observation. In the vast percentage of such cases it is unlikely that the reaction is one of true anaphylaxis. However, the theory has been advanced by Wolff-Eisner that some drugs may alter the blood or tissue proteins of certain individuals in such a way that they act as foreign proteins, thus sensitizing the individual. It is barely possible that some such conditions might, at times, occur, in which case the reaction would be anaphylactic.

Other Allergies. In veterinary practice the mallein test for glanders and the tuberculin test for tuberculosis are highly important diagnostic procedures. These tests depend on the production of an *allergic reaction* in animals affected with glanders or tuberculosis when injected with mallein or tuberculin. By some authorities the mallein and tuberculin reactions have been held to be true anaphylaxis. Such reactions are, obviously, not manifestations of true anaphylaxis as we know the term. The exact mechanism of these reactions has not been satisfactorily determined, and, inasmuch as they are not of a true anaphylactic nature, the best we can do is to classify them as allergic reactions.

[9] Wells, H. G., The Chemical Aspects of Immunity, 1925, pub. by Chemical Catalog Co., New York, N. Y.

PART IV

PATHOGENIC ORGANISMS OF THE CLASS SCHIZOMYCETES

CHAPTER XI

Order—Eubacteriales, Family—Coccaceae, Tribe—Streptococceae. Organisms of the Genus Streptococcus

One of the most perplexing problems in bacteriology is the differentiation and satisfactory classification of streptococci. An enormous amount of work looking towards a satisfactory means for the identification and classification of this group of organisms has been done and a number of methods have been advocated but the goal has not as yet been reached.

While streptococci were undoubtedly observed by some of the early bacteriologists, the first record of isolation of this type of organism in pure culture was that of Fehleisen, in 1883, who isolated a streptococcus from a case of human erysipelas. Shortly following this (1884) Rosenbach isolated from an abscess in man a streptococcus which he termed "Streptococcus pyogenes."

At first it was thought that the various streptococci present in different conditions or diseases were specific for the particular disease or lesion they were present in. It was soon found, however, that some of the streptococci isolated could produce a variety of pathological conditions. This then gave rise to a tendency to consider all pathogenic streptococci as possibly the same species. This latter view became somewhat modified as more knowledge of streptococci was gained. Thus, one common type of streptococci (Streptococcus pyogenes) came into prominence and has been found capable of producing a great variety of conditions, while to a limited extent, other types of streptococci have been linked with certain maladies either as specific causes or secondary factors.

Early attempts at classification merely took into consideration morphology and behavior on blood-agar culture media. On this basis Schottmüller, in 1903, designated all long chain streptococci of the so-called "pyogenes" type which produced hemolysis on blood plates as "Streptococcus hemolyticus." By the term "Streptococcus mitior" or "Streptococcus viridans" he designated the less virulent, usually short chain streptococci, colonies of which have a green halo about them on blood agar plates. He also recognized a third type which, because of the mucoid nature of its growth, he termed "Streptococcus mucosus." This latter organism has since been found to belong to the pneumococci rather than the streptococci.

Attempts have been repeatedly made to classify streptococci according

to their action on various carbohydrates. The results have been very discouraging. Holman proposed a classification which for a while was used by a considerable number of bacteriologists. He separated streptococci into two main groups according to whether they were hemolytic or non-hemolytic and then proceeded to further classify them according to their action in litmus milk and media containing lactose, mannite and salicin.

A rather broad classification of streptococci which is of considerable value is that prepared by Smith and Brown. They recognize three types of streptococci—"Alpha," "Beta," and "Gamma."

The *Alpha type* includes those commonly referred to as the *viridans type.* They produce no definite hemolysis but the colonies are surrounded by a greenish discolored zone due to the production of methemoglobin. More or less hemolysis does occur with some of these strains but it is not readily discernible because of the greenish discoloration of the medium around the colonies.

The *Beta type* designates streptococci which are markedly hemolytic, producing a definite, clear-cut zone of hemolysis around the colonies on blood agar plates.

Under *Gamma type* are grouped those streptococci which are non-hemolytic and which do not produce a greenish discoloration of the blood agar. A number of saprophytic strains fit into this group.

In cultivating streptococci to test them for hemolytic properties, nutrient veal agar having a reaction of approximately pH 6.8 should be used as a base for the test medium. It is prepared in the usual manner using 1 part of veal to 2 parts of water and adding 0.50 per cent sodium chloride, 1 per cent peptone and 1.50 per cent agar. Brown recommends putting this base up in tubes, placing 12 cc. in a tube. When ready for use a number of tubes are heated in the Arnold sterilizer and when the agar has melted they are transferred to a water bath maintained at 45°C. After allowing 15 or 20 minutes for the tubes and contents to cool down to the temperature of the water bath, 0.60 cc. of sterile, defibrinated rabbit, horse, or human blood is added to each tube and those to be inoculated are returned to the water bath. A loopful of a young culture of the streptococcus to be studied is inoculated into the tube of the medium and the same poured into a sterile, standard-size Petri dish. The results are observed after 48 hours incubation.

While the most accurate results are obtained by preparing the blood medium as described above immediately before use, for ordinary purposes it can be prepared as outlined and poured into sterile Petri dishes for subsequent use as streak plates and if reasonably fresh, satisfactory results are obtained.

The Committee of the Society of American Bacteriologists has attempted to group streptococci according to their action on blood agar and serological reactions, their action on carbohydrates being given secondary consideration.

There has been considerable controversy as to the relationship of streptococci isolated from man and those from the lower animals. Some authorities have held that Streptococcus pyogenes is a common parasite of both man and lower animals. Others maintain that such organism is primarily a human type, lower animals being resistant to same. Some investigators have held that the streptococcus of bovine mastitis is a common cause of septic sore throat in the human family while others disagree entirely with such view. The weight of evidence indicates that man is relatively refractory to the streptococci producing ailments in lower animals and, conversely, the lower animals are not commonly affected by streptococci of human origin. It is undoubtedly true, however, that rarely cases of streptococcic infection in man are due to streptococci of animal origin and, on the other hand, that lower animals occasionally are infected with streptococci of human origin.

Milk or other animal products handled by man may be contaminated with streptococci of human origin and through such products the infection may be transmitted to other persons. It can be readily understood how, under such circumstances, the organism might be considered of animal origin.

In a general way, as Brown[1] has pointed out, streptococci of bovine origin are less hemolytic than those of human origin. This can be demonstrated by quantitative hemolysin tests carried out in test tubes.

Streptococcus mastitidis, which has been considered by a number of investigators as identical with S. pyogenes, will as shown by Avery and Cullen,[2] and Ayers and Mudge,[3] produce a lower "final pH" in cultures than that produced by Streptococcus pyogenes. Ayers and Mudge have also shown that the bovine Streptococcus mastitidis will ordinarily hydrolize sodium hippurate whereas Streptococcus pyogenes will not.

BIPRODUCTS OF STREPTOCOCCUS METABOLISM

Hemolysin. The hemolysin produced by the hemolytic strains of streptococci was first reported in 1895 by Marmock.[4] At that time investigators considered that the virulence of streptococci varied in direct proportion with their ability to produce hemolysin.

[1] Brown, Jour. Exp. Med., vol. 31, 1920, p. 35.
[2] Avery and Cullen, Jour. Exp. Med., vol. 29, 1919.
[3] Ayers and Mudge, Jour. Inf. Dis., vol. 31, 1922.
[4] Marmock, An. del'Inst. Pasteur, 1895.

Streptococcus hemolysin is very unstable. Under artificial conditions it is produced early in the culture of streptococci, the most being obtained from cultures, 12 to 16 hours old. As fluid cultures become older the hemolysin rapidly diminishes, frequently disappearing entirely after 36 to 42 hours cultivation. Media containing serum or ascitic fluid are particularly well suited for hemolysin production. The exact nature of streptococcus hemolysin is not known. Attempts to produce neutralizing antibodies through injection of the hemolysin into animals have failed. In this connection, however, it should be mentioned that occasionally a normal serum will be found which will neutralize streptococcus hemolysin.

The simplest test to determine whether or not a strain of streptococcus is hemolytic is to inoculate blood-agar plates with the organism, incubate them for 24 to 48 hours and examine for the presence or absence of a zone of hemolysis around the colonies. Where it is desired to make a quantitative test, serum-broth cultures grown for 16 to 18 hours are centrifugalized at high speed for one-half hour and the supernatant fluid drawn off and added in variable amounts to tubes containing 0.50 cc. of a 3 per cent suspension of washed sheep corpuscles in physiological salt solution. The rack containing the tubes is then placed in the water bath at 37.5°C. for one hour at the end of which time it is removed and the smallest amount of the culture causing complete hemolysis of the red blood cells noted.

In studying streptococcus hemolysin young cultures (16 to 18 hours old) can be filtered through Berkefeld filters to render them free of bacteria but the filtrate will be found to have lost a considerable amount of hemolysin as a result of the process.

Streptococcus leucocidin. Virulent strains of streptococci produce a substance which has the property of preventing or inhibiting phagocytosis through its action on the white cell. This product known as "streptoleucocidin" was first demonstrated with streptococci by Ruediger[5] in 1905.

Like hemolysin, leucocidin is produced during the early hours of cultivation, the greatest amount with a given strain of streptococcus is present after 12 to 18 hours cultivation. It differs from the hemolysin in that it is destroyed by a temperature of 60°C. for one-half hour.

The production of neutralizing substances has been demonstrated in the serum of animals injected with leucocidin. Smaller quantities of neutralizing substances have been found present in normal sera.

According to Nakayama[6] the amount of leucocidin produced by a strain of streptococcus is in direct proportion to its virulence. For measuring

[5] Ruediger, A. M. A. Jour., 1905, 44, p. 198.
[6] Nakayama, Jour. Inf. Dis., 27, 1920, 86.

the amount of leucocidin Nakayama describes a method which involves the following procedure:

Obtain an exudate rich in leucocytes by injecting rabbits intrapleurally with aleuronat or guinea pigs intraperitoneally with peptone and prepare a suspension of the white cells as outlined for opsonic index determination. Have ready a solution of methylene-blue made by mixing 1 cc. of saturated solution of methylene-blue, 20 cc. absolute alcohol and 29 cc. of distilled water. Varying amounts of the basic suspension of leucocytes in 0.90 per cent sodium chloride solution are placed in test tubes and the total volume of each tube brought up to 2 cc. with a 0.90 per cent salt solution. Two drops of the methylene-blue solution are added to each tube. The surface of the fluid is then layered with liquid petrolatum and the tubes placed in the incubator at 37°C. for two hours. Reduction of the methylene-blue is indicated by the solution becoming colorless. Where reduction has not occurred the solution remains green. The smallest amount of leucocyte suspension causing complete reduction of the methylene-blue is determined. Twice the amount of leucocyte suspension causing reduction of the methylene-blue is placed in each of a series of test tubes and varying amounts of the fluid to be tested are added to the different tubes. The tubes are then placed in the incubator at 37°C. for $1\frac{1}{2}$ hours to give any leucocidin present an opportunity to act on the leucocytes. At the end of this incubation period 2 drops of the methylene-blue solution are added to each tube and liquid petrolatum layered over the surface of the fluid. The tubes are again incubated for two hours when they are removed and the results recorded, loss of color indicating reduction, persistence of the green color signifying no reduction.

Streptococcus Toxin. Great impetus has been given the study of streptococci for toxin production since the recent work of the Dicks[7] with cultures of streptococci isolated from cases of scarlet fever. Evidence to date tends to confirm their reports of the presence of a specific toxin in suitable cultures of scarlatina streptococci. This is further supported by ability to prepare a neutralizing antitoxin. These findings have a direct bearing on the whole question of biproducts of streptococci generally and already have led to the initiation of work with streptococci commonly found in other diseases with a view to demonstrating possible toxin production by other types. From time to time various investigators have reported toxin production by various strains of streptococci but subsequent work by other investigators generally resulted in conflicting reports so that this possible property of streptococci was not established.

[7] Dicks, Jour. A. M. A., 1924, 82, p. 265.

The findings of the Dicks, which have been receiving rather general confirmation, tend to indicate that, after all, some of the claims for toxin production by streptococci may not have been far wrong. More illuminating knowledge on this subject is possibly not far distant.

Immunity to Streptococci. Apparently but little immunity to subsequent infection results from invasions of streptococci. There is evidence, however, which tends to indicate that an animal's immunity to a given type of streptococcus can be substantially raised by giving rather large quantities of bacterin over a period of time. Further anti-streptococcic serum of demonstrable protective value can be prepared with some strains of streptococci by injecting horses first with killed and later gradually increasing doses of living culture. The use of such biological products in some of the streptococcic infections is, therefore, of value. In the administration of serum in the treatment of streptococcic infections best results are obtained in subacute or localized conditions. Early administration of the serum is imperative, especially in the more serious conditions, if any results are to be expected from the same. Once an active, more or less generalized, streptococcic infection gets well started, the results from serum are usually discouraging.

The specifications for the genus Streptococcus as given by the Committee of the Society of American Bacteriologists are as follows:

> Chiefly parasites. Cells in pairs or in short or long chains, never in packets. Generally Gram-positive. Capsules rarely formed. Do not form zoogleal masses. Grow as effused, translucent, often small isolated colonies on agar streak. In stab cultures little surface growth is developed. Many carbohydrates are fermented with formation of acid, but inulin is rarely attacked. Generally fail to liquefy gelatin or reduce nitrates. Some species lake blood, others produce methemoglobin, while a smaller number are without action on blood.

Under the Genus Streptococcus the following species of importance or interest in veterinary medicine are considered: Streptococcus equi (Schütz), Streptococcus mastitidis, Streptococcus pyogenes, Streptococcus lactis.

STREPTOCOCCUS EQUI (SCHÜTZ)

In 1888 Schütz described Streptococcus equi as the etiological factor in equine "strangles" or distemper. That this organism is the actual primary cause of strangles has, from time to time, been questioned by some investigators. However, it has been found invariably present in cases of the disease and at present is rather generally accepted as the causative agent.

In strangles the organism is present in the affected glands and the nasal

discharge. The submaxillary glands are chiefly involved although the infection may extend to other glands and tissues. Streptococcus equi can be obtained in pure culture from pus evacuated with aseptic precautions from the submaxillary glands.

Aside from its rôle as the primary factor in strangles, Streptococcus equi is an important secondary agent in contagious pleuro-pneumonia of horses. In such disease it is found in the lungs, pleural exudate, nasal discharge and in some cases it can be isolated from the blood. In culturing the blood a considerable quantity (15 to 25 cc.) should be bled with aseptic precautions into a flask containing 150 to 200 cc. of dextrose bouillon.

In contagious pleuro-pneumonia Streptococcus equi is commonly found associated with Pasteurella equisepticus.

In the affected lung tissue Streptococcus equi commonly appears singly and in pairs. This characteristic led some investigators to consider the organism a diplococcus. By some it has thus been termed "Diplococcus pneumoniae" and considered as the cause of the disease.

Morphology and Staining Characteristics. Streptococcus equi usually occurs in long, twisted chains made up of from 40 to 65 or more organisms varying from 0.4 to 1 micron in diameter. Short chains, diplococci and even single cells may be observed. The individual cells frequently vary from the true spherical form, some appearing oval, others angular. They multiply by binary division in a single direction. They are non-motile and do not form spores. Some particularly virulent strains occasionally suggest that they possess a capsule.

This organism stains well with all the ordinary aniline dyes and is Gram-positive. A diluted solution of carbol-fuchsin (1:10) or Loeffler's methylene-blue can be used for demonstrating the organism in pus, exudates, etc., with very satisfactory results.

Cultural Characteristics. Streptococcus equi can be cultivated with little difficulty. While it will grow on ordinary media, those containing blood, serum or carbohydrates favor its propagation. Glucose serum agar, blood agar, glucose broth, etc., are very satisfactory. Chain formation is best demonstrated in fluid media.

The organism is an aerobe and facultative anaerobe. It grows best at 35° to 37.5°C. but will develop slowly at room temperature. Media of a slightly alkaline reaction are best suited for its propagation. The growth of streptococci slows up and finally stops when a considerable quantity of acid has been produced.

Agar. On agar, fine, pin-head or smaller-sized, grayish-white colonies develop. They are firmly attached to the agar and do not tend to coalesce.

When cultivated on *serum agar* the growth is more luxuriant, the colonies appear translucent and glistening and after several days' growth tend to coalesce. On *blood agar* a zone of hemolysis surrounds the colonies.

Gelatin. In gelatin stab cultures Streptococcus equi produces a fine, white, punctiform growth along the stab. There is no liquefaction of the medium.

FIG. 22. STREPTOCOCCUS EQUI

Bouillon. Broth cultures become slightly turbid, but, as is more or less characteristic of long chain streptococci, fine flocculi develop and soon gravitate to the bottom of the tube leaving the medium above clear.

Litmus Milk. Litmus milk is rendered acid.

Potato. No growth occurs on potato.

Fermentable Media. Salicin is fermented by Streptococcus equi. Lactose and mannite are not fermented.

Pathogenicity. Intraperitoneal injections of recently isolated strains of Streptococcus equi into mice produce death in 24 to 48 hours. Rabbits are sometimes killed by intravenous injections of some strains. Other laboratory animals are more or less refractory.

Resistance. Streptococcus equi is a somewhat resistant organism. In pus or exudates it will resist drying for several weeks and will survive exposures to temperatures up to 60° to 62°C. for rather long periods of time. Freezing does not affect it. It is killed by subjecting it to a temperature of 75°C. for 30 minutes. Eighty-five degrees centigrade for 15 minutes destroys it; boiling is effective immediately. It succumbs readily to the action of the common disinfectants. A 5 per cent solution of phenol, 1:1000 bichloride of mercury, 3.5 per cent solution of liquor cresolis compound and 2 per cent formalin destroy the organism within 10 minutes.

STREPTOCOCCUS MASTITIDIS (STREPTOCOCCUS AGALACTIAE CONTAGIOSAE, STREPTOCOCCUS PYOGENES BOVIS, STREPTOCOCCUS OF BOVINE MASTITIS)

While bovine mastitis may result from infection with any one of several different organisms, by far the most common type is that caused by a streptococcus. Various investigators, in reporting work done with bovine mastitis in which a streptococcus was considered the etiological factor, have either given the organism a special name or referred to it as a strain of Streptococcus pyogenes. Thus we find Streptococcus mastitidis (Guillebeau), Streptococcus agalactiae contagiosae (Kitt), Streptococcus pyogenes bovis, Streptococcus of bovine mastitis, etc., named as the inciting agents in various outbreaks of mastitis among dairy cows. The same type of streptococcus was probably the causative factor in all or at least a large percentage of such cases, inability to satisfactorily differentiate and classify streptococci being responsible for the variety of terms.

Morphology and Staining Characteristics. Streptococcus mastitidis usually forms long chains of from 50 to 75 spheres. Some strains grow in shorter chains. The individual organisms are usually from 0.5 to 1 micron in diameter but they vary considerably in size. Streptococcus mastitidis is readily stained with the ordinary aniline dyes and is Gram-positive.

Cultural Characteristics. Streptococcus mastitidis is an aerobe and facultative anaerobe. It grows well at the usual incubator temperature, less rapidly at room temperature.

Agar. On agar incubated at 37.5°C. the organism develops fine, convex, pin-head sized, slightly grayish, glistening colonies. Growth on blood agar reveals evidence of hemolysin production.

Gelatin. Gelatin cultures slowly develop very fine, white colonies along the line of the streak. There is no liquefaction of the medium.

Bouillon. In broth of pH 7.6 to 7.8 the organism grows well, forming long chains which form flocculi and settle to the bottom of the tube. There is usually but slight tendency towards clouding of the medium.

Litmus Milk. Litmus milk is coagulated within 24 to 36 hours followed by partial decolorization.

Potato. Potato is not a suitable medium for this organism.

Fermentable Media. Lactose and dextrose are usually fermented whereas

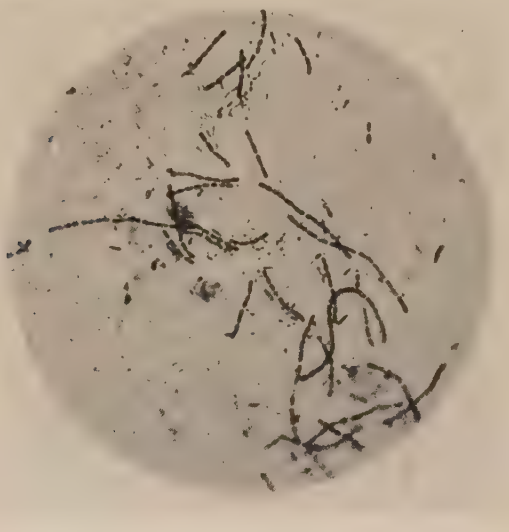


FIG. 23. STREPTOCOCCUS MASTITIDIS IN MILK

mannite, inulin and raffinose are not. Some strains ferment salicin, others do not.

Ayers and Mudge[8] consider "final pH" values of dextrose-yeast peptone broth cultures and the ability or inability of the streptococcus to hydrolyze sodium hippurate into benzoic acid and glycocoll, as valuable for the differentiation of Streptococcus mastitidis and Streptococcus pyogenes of human origin. As first pointed out by Ayers and Rupp,[9] Streptococcus mastitidis will hydrolyze sodium hippurate while Streptococcus pyogenes will not.

[8] Ayers and Mudge, Jour. Inf. Dis., vol. xxxi, p. 40, 1922.

[9] Ayers and Rupp, Ibid., vol. xxx, p. 388, 1922.

The final pH that is reached when growth ceases in the peptone broth culture containing yeast and dextrose is around 4.5 with Streptococcus mastitidis while Streptococcus pyogenes ceases growth with a final pH of approximately 5.5.

The medium for the final pH test contains the following:

Peptone	10 grams
Yeast (Cerevisine, a French preparation)	10 grams
Distilled water	1000 cc.

Adjust reaction to pH 7.5 and add 1 per cent dextrose

Cultures are grown in this medium for one week when the reaction is determined colorimetrically.

For the sodium hippurate test Ayers and Rupp recommend a medium consisting of the ingredients indicated below. Such medium is satisfactory for the propagation of most streptococci. However, if cultures are found which will not grow in this medium ordinary beef broth medium, with or without dextrose, may be employed.

Peptone-Pepsin Medium

Peptone	10.00 grams
Pepsin	5.00 grams
Calcium chloride	0.03 gram
Sodium hippurate	10.00 grams
Distilled water	1000.00 cc.
Ferric chloride (1 per cent solution)	1 drop

The reaction of this medium is adjusted to pH 7.1 with a normal solution of NaOH.

Streptococci under examination are grown in the above described medium for one week when the cultures are tested to determine whether or not hydrolysis of the sodium hippurate has occurred. Ayers and Rupp employed several methods for such determination but indicate that the ferric-chloride test is the most delicate.

When ferric chloride is added to the medium, the protein, any unsplit hippurate, and any benzoate present as a result of hydrolysis of the hippurate, are precipitated. In an excess of ferric chloride the protein and hippurate redissolve leaving only the benzoate. Thus, in testing for hydrolysis, a definite amount of a ferric chloride solution must be added to a definite amount of culture medium. When the peptone-pepsin medium is employed 0.50 cc. of a 7 per cent ferric chloride solution is added to 2 cc. of the medium and the same thoroughly shaken and allowed to stand

5 or 10 minutes. If the sodium hippurate has been split into benzoate and glycocoll an insoluble precipitate is demonstrable. Ayers and Rupp state that where 20 per cent or more of the hippurate has been split the precipitate is well marked whereas only a slight turbidity occurs when less of the hippurate is hydrolyzed. Streptococcus mastitidis ordinarily causes hydrolysis of all of the hippurate present, the reaction thus being well marked.

When beef infusion broth is employed in place of the peptone-pepsin medium, a 12 per cent ferric chloride solution containing 0.20 to 0.25 per cent concentrated HCl is used as the test reagent. A longer period of time is required for redissolution of non-specific precipitates in negative tests.

Resistance. From the standpoint of tenacity Streptococcus mastitidis does not differ materially from other pathogenic streptococci. Temperatures around 70° to 75°C. destroy it in 30 to 45 minutes, 85°C. in 15 minutes, boiling immediately. The common disinfectants in the usual strength solutions kill the organisms within 15 minutes.

Carriers. Streptococcus mastitidis is frequently carried for long periods of time in the apparently normal udder by healthy cows. Such animals may later become affected with mastitis themselves or the infection may be spread by milkers to less resistant cows and give rise to mastitis.

Biological Therapy and Prophylaxis. Various investigators have reported good results from bacterins in the treatment and prevention of bovine mastitis. For the preparation of bacterins the reader is referred to the chapter on that subject, page 464.

STREPTOCOCCUS PYOGENES

As already indicated Streptococcus pyogenes is not ordinarily a common parasite of the lower animals. However, in such animals it may be occasionally found in various types of suppurative processes.

Morphology and Staining Characteristics. Streptococcus pyogenes does not differ appreciably from any of the other streptococci in morphology and staining characteristics. It occurs in chains varying from 2 or 3 to 75 or more organisms. Frequently, the chains appear to be made up of pairs of diplococci arranged end to end. The individual cocci appear round, oval and occasionally some are found that have a somewhat irregular, flattened appearance. Streptococcus pyogenes is non-motile, stains well with the usually employed aniline dyes and is Gram-positive.

Cultural Characteristics. Streptococcus pyogenes is an aerobe and facultative anaerobe. It grows best at a temperature of 36° to 38°C. but will propagate less satisfactorily at lower temperatures. Media of a slightly alkaline reaction are better suited for its growth than acid media. Blood,

blood serum, and glucose added to various media materially favor the growth of this organism.

Agar. On agar plates fine, pin-point-like, transparent colonies develop within 24 hours. These later become a trifle larger and more opaque, appearing grayish-white. On glucose, serum or blood agar the growth is more

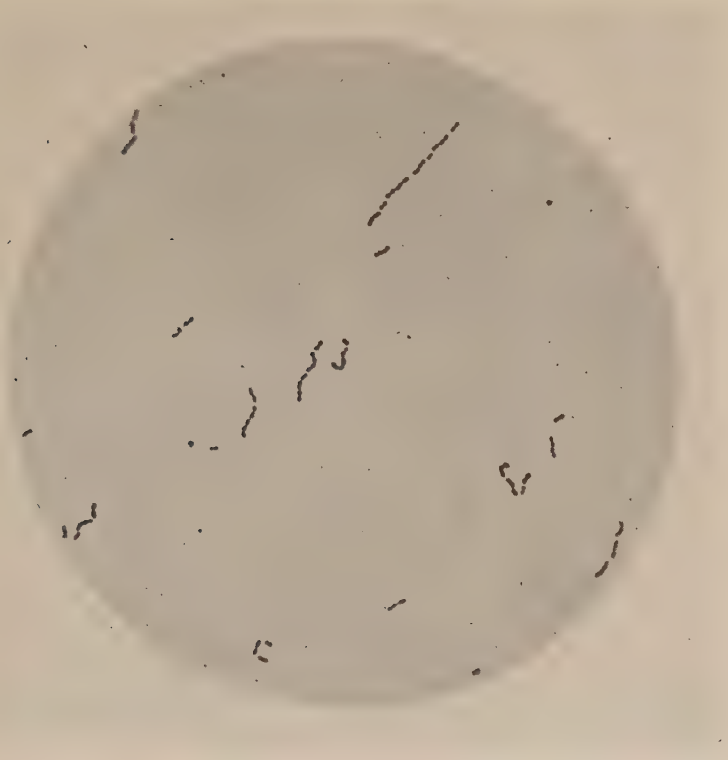


Fig. 24. Streptococcus pyogenes

luxuriant and on the latter medium the colonies are surrounded by a zone of hemolysis.

Gelatin. Colonies on gelatin plates are similar to those on agar. There is nothing characteristic about the growth in gelatin stab-cultures. The medium is not liquefied.

Bouillon. Broth cultures may appear slightly turbid in the beginning but they usually clear up.

Litmus Milk. This medium is rendered acid and may or may not be coagulated.

Potato. Ordinarily no growth occurs on this medium.

Fermentable Media. Streptococcus pyogenes produces acid in media containing lactose, salicin and sucrose.

Resistance. Streptococcus pyogenes will resist temperatures below 60°C. for a considerable period of time. A temperature of 75°C. will destroy it in 30 minutes; 85°C. in 10 to 15 minutes, and boiling immediately. The various chemical disinfectants commonly employed are effective in the usual strength solutions in 15 minutes to one-half hour.

Pathogenicity. Mice are susceptible to intraperitoneal injections of Streptococcus pyogenes and rabbits to intravenous injections. The organism loses its virulence rapidly when maintained on artificial culture media.

STREPTOCOCCUS LACTIS

While non-pathogenic, Streptococcus lactis is briefly mentioned here because it is the common cause of the souring of milk and it may possibly be mistaken by casual observers as of pathological significance.

Streptococcus lactis does not produce hemolysis nor green coloration of blood agar. The chains are usually much shorter (3 or 4 spheres) than the virulent types of streptococci. Some of the cells appear elongated with tapering ends.

The organism is an aerobe and facultative anaerobe. It grows rapidly at incubator temperature but also grows well at lower temperatures. It is readily stained by aqueous solutions of the ordinary aniline dyes and is Gram-positive. The organism grows well on agar, particularly blood or serum agar, in broth, milk, and somewhat less so in gelatin. Litmus milk is rendered markedly acid and coagulated. In this connection it should be remembered that with Streptococcus mastitidis coagulation occurs first and is followed by incomplete reduction of the litmus. With Streptococcus lactis the color reduction precedes curdling and is complete.

Streptococcus lactis produces acid in media containing lactose, salicin and mannitol.

CHAPTER XII

Order—Eubacteriales, Family—Coccaceae (Continued), Tribe—Micrococceae. Organisms of the Genus Staphylococcus

The characteristics of the members of this genus, as given by the Committee of the Society of American Bacteriologists are as follows:

Usually parasitic, cells occur singly and in pairs and in irregular groups, rarely in packets. Usually Gram-positive. Growth fair to good on the surface of artificial media. As a rule carbohydrates are fermented with the formation of acid. Gelatin commonly liquefied. Nitrates may or may not be reduced. (Produce hemolysis on blood agar.) Pigment white or orange, or less commonly lemon-yellow.

The staphylococci are important members of the pyogenic or pus-producing group of bacteria. They are found widely distributed in nature and are common inhabitants of the skin and mucous membranes of animals. Pathogenic forms are capable of producing a variety of conditions. Abscesses, fistulae, wound infections, various inflammatory and suppurative processes, septicemia, etc. are very frequently due to the activities of staphylococci.

There are three types of staphylococci of importance in veterinary medicine. These are Staphylococcus aureus, Staphylococcus albus, and Staphylococcus citreus. Staphylococcus aureus produces a golden-yellow pigment, Staphylococcus albus colonies are white, while Staphylococcus citreus produces a lemon-yellow pigment.

Staphylococcus aureus is the type most frequently found and is the most virulent. Staphylococcus albus is of less frequent occurrence and is somewhat less virulent. Staphylococcus citreus is the least common of the pathogenic Staphylococci and its virulence is inferior to that of the other two types.

Pigments of Staphylococci. The pigment produced by staphylococci is, according to the work of Schneider,[1] a form of lipochrome. It is secreted by the bacterial cell to which it remains adherent unless the organism be placed in a fat solvent. Thus, it can be extracted by ether, chloroform, etc.

The different staphylococcus pigments are apparently constant for the different species. This has, obviously, been of tremendous value in classifying this group of organisms.

[1] Schneider, Arb. a. d. bakt. Inst., Karlsruhe, 1894.

Staphylococcus Hemolysin. The amount of hemolysin produced by staphylococci varies with different strains. In a very rough way the more virulent strains produce the most hemolysin. Unlike the streptococcus, the staphylococcus does not produce the maximum amount of hemolysin during the first few hours (12 to 16) of its growth. The peak of hemolysin production with staphylococcus cultures is not reached until after 10 to 14 days' incubation.

Staphylococcus aureus is the most important of the staphylococci as a hemolysin producer. Staphylococcus albus, as a rule, is only a feeble producer of hemolysin. Staphylococcus citreus rarely produces hemolysin.

Staphylococcus hemolysin is destroyed by twenty minutes exposure to a temperature of 56°C.

Staphylococcus Leucocidin. Staphylococcus leucocidin, a substance similar to that already described in connection with streptococci, was discovered as a bi-product of metabolism of the staphylococcus before it was demonstrated with streptococci. Van de Velde,[2] in 1894, was the first to demonstrate that virulent staphylococci were able to produce a substance which had the ability to destroy leucocytes. He termed this substance "leucocidin" and found that it was not only formed within the animal body but could be produced in vitro as well.

Tests for leucocidin can be made in the manner described for the demonstration of a similar substance in streptococcus cultures (see page 131).

Immunity to Staphylococci. Immunity to staphylococci infection can be increased by the use of properly prepared bacterins. Such agents frequently give excellent results in the treatment of chronic or localized staphylococcic infections. In such cases autogenous bacterins are better than stock bacterins.

An anti-staphylococcus serum can be prepared by immunizing horses with gradually increasing doses of killed, and finally living, staphylococci. Such serum is of value in some cases of staphylococcus infection.

STAPHYLOCOCCUS AUREUS (STAPHYLOCOCCUS PYOGENES AUREUS)

Morphology and Staining Characteristics. Staphylococcus aureus is a spherical organism, the average of which vary between 0.7 and 1.0 micron in diameter. They are usually found grouped in irregular masses or grape-like clusters which characteristic gave rise to the name "staphylococcus." In exudates, pus, etc., pairs or even single cells may be observed. Some of the cells in close contact with others, may present a flattened border at

[2] Van de Velde, La cellule, 1894, vol. x.

the point where they touch. The organism possesses no true motility but shows very definite molecular (Brownian) activity. It is not encapsulated and does not produce spores.

Staphylococcus aureus is readily stained with aqueous solutions of the various aniline dyes and is Gram-positive.

Cultural Characteristics. Cultures of Staphylococcus aureus will grow between rather wide temperature limits (10° to 42°C.). The optimum temperature is less than that of the body (32° to 34°C.). The organism is an aerobe and facultative anaerobe and grows well on most all of the common types of culture media. While a slightly alkaline reaction is most

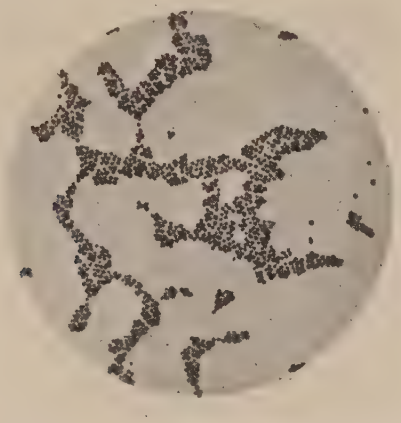


FIG. 25. STAPHYLOCOCCUS AUREUS

conducive to good growth, there is no difficulty in cultivating it on slightly acid media.

Agar. On agar plates small, round, slightly raised, glistening colonies develop within 24 hours. These are at first grayish-white but as the culture becomes older the characteristic golden-yellow color develops.

In slant and stab cultures a grayish-white growth develops. This subsequently becomes golden-yellow where oxygen is present. The growth in the butt does not take on the characteristic color because pigment is produced only in the presence of oxygen.

The organism grows very well on blood agar and will produce hemolysin. There is a rather marked variation, however, in the amount of hemolysin produced by different strains of Staphylococcus aureus.

Gelatin. The growth on gelatin plates is rather characteristic. Plates inoculated and left at room temperature will show, within 48 hours, small, glistening, slightly raised colonies of a grayish-white appearance. These gradually become darker in color until the characteristic golden-yellow hue is evident. Liquefaction of the gelatin can be noted around the colonies after 48 hours growth. Careful observation will reveal the colonies lying in sharply defined, saucer-like depressions resulting from liquefaction. As the growth continues the area of liquefaction around each colony spreads and in time becomes confluent with other areas.

In gelatin stabs a grayish-white growth appears along the line of the stab and this is soon followed by funnel-shaped liquefaction of the medium. Liquefaction proceeds rather rapidly and in the course of a few days is complete with the organisms deposited at the bottom of the tube.

The staphylococcus protein ferment causing the liquefaction of gelatin is known as "gelatinase." It can be demonstrated in bacteria-free filtrates of staphylococcus cultures.

Bouillon. The organism grows rapidly in broth, uniformly clouding the medium. In the majority of cases a thin pellicle appears after two or three days of incubation. As the culture ages a heavy, mucoid sediment accumulates at the bottom of the tube.

Litmus Milk. Milk is acidified and coagulated by Staphylococcus aureus. The period required for coagulation is variable, 8 days elapsing in some instances before the same occurs.

Potato. The organism grows well on potato producing its characteristic pigment.

Peptone Solution. Indol is not produced when grown in Dunham's peptone solution.

Nitrate Solution. Growth in such solution demonstrates ability of the organisms to reduce nitrates to nitrites.

Loeffler's Serum Media. Staphylococcus aureus grows well on this medium and slowly brings about its liquefaction.

Fermentable Media. Acid is produced in media containing lactose, dextrose, saccharose, sucrose, and mannitol. No acid is produced from salicin, raffinose or inulin.

Resistance. Staphylococci resist drying for a number of weeks and in a dry state will tolerate rather high temperatures (60° to 70°C.) for considerable periods. A temperature of 80°C. for 30 minutes is necessary to insure destruction of the organism in such state. When suspended in fluid a temperature of 60°C. for 20 minutes will destroy staphylococci. Bichloride of mercury, phenol, liquor cresolis compound, formalin, etc.,

are efficacious in the usual strength solutions, destroying the organism within one-half hour.

STAPHYLOCOCCUS ALBUS (STAPHYLOCOCCUS PYOGENES ALBUS)

This organism is similar in its morphological and cultural characteristics to Staphylococcus aureus, with the exception that it does not produce the brown pigment. Further, its ability to produce hemolysin and leucocidin is less than that of the aureus type. Staphylococcus albus, as has been pointed out, is somewhat less virulent than Staphylococcus aureus. Its resistance to heat, disinfectants, etc., is similar to that of Staphylococcus aureus.

STAPHYLOCOCCUS CITREUS (STAPHYLOCOCCUS PYOGENES CITREUS)

Staphylococcus citreus differs from the other staphylococci in that it produces a lemon-yellow pigment. It rarely produces hemolysin and but small amounts of leucocidin. It is the least virulent of the pathogenic organisms of this group and is not of very common occurrence.

CHAPTER XIII

Order—Eubacteriales (Continued) Family—Spirillaceae. Organisms of the Genus Vibrio

The family "Spirillaceae" is not divided into tribes but is made up of two genera—"Vibrio" and "Spirillum." This family is not of particular importance in veterinary medicine. At the present time there is but one organism belonging to this group worthy of mention. That is Vibrio metchnikovi, the causative agent of a cholera-like disease of chickens. As indicated by the name, this organism belongs to the genus "Vibrio."

The specifications of this genus as given by the Committee of the Society of American Bacteriologists are as follows:

> Cells short, bent rods, rigid, single or united into spirals. Motile by means of a single (or rarely, two or three) polar flagellum, which is usually relatively short. Many species liquefy gelatin and are active ammonifiers. Aerobic, facultative, anaerobic. No endospores formed. Usually Gram-negative. Water forms; a few parasites.

VIBRIO METCHNIKOVI

The first report on this organism was by Gamaleia[1] in 1888. He published the results of an investigation of a rapidly fatal disease of chickens in Odessa in which he found the etiological factor to be a cholera-like vibrio which he named "Vibrio metchnikovi." The disease is spoken of as vibrio cholera of chickens, vibrio septicemia or avian vibrio gastro-enteritis. It is characterized by a marked enteritis accompanied by a profound diarrhea.

The organism can be demonstrated in large numbers in the involved portions of the intestinal tract and in the feces of affected birds. The affection usually terminates fatally within 48 to 72 hours.

Vibrio metchnikovi is in many respects similar to the vibrio of Asiatic cholera of man but there is no doubt of its being entirely distinct from such organism.

Morphology and Staining Characteristics. Vibrio metchnikovi occurs as a comma-shaped rod measuring about 1.8 microns in length and 0.4 to 0.5 micron broad. In addition to the typical comma-shaped forms a more chubby, almost coccoid type occurs and in cultures grown for several days spirals made up of several organisms adhering to one another may be dem-

[1] Gamaleia, An. de l'Inst. Pasteur, 1888, ii, 482.

onstrated. The organism possesses a single polar flagellum and is actively motile.

Vibrio metchnikovi stains satisfactorily with aqueous solutions of the various aniline dyes but dilute carbol-fuchsin is particularly valuable in demonstrating it. It is Gram-negative.

Cultural Characteristics. Vibrio metchnikovi can be readily cultivated on most of the ordinary media. It is an aerobe and facultative anaerobe. It will grow at room temperature but develops more rapidly when incubated at regular incubator temperature.

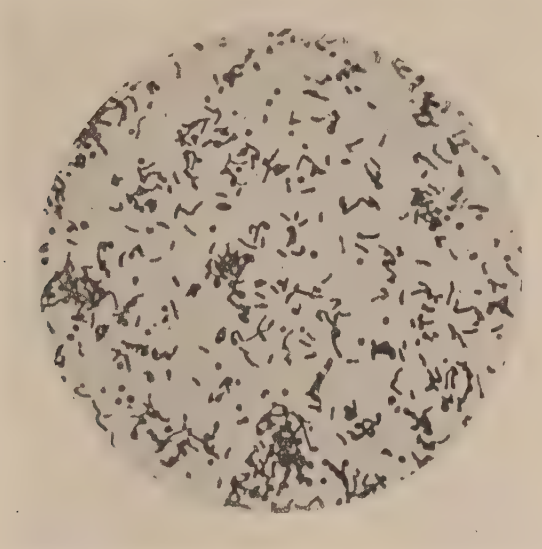


FIG. 26. VIBRIO METCHNIKOVI

Agar. The organism on agar plates produces small, round, slightly raised, glistening colonies of a faintly yellowish color. On agar slants a moist, glistening, yellowish growth develops.

Gelatin. On gelatin plates left at room temperature small, pin-head size colonies develop. They are slightly yellowish and under slight magnification are found to be granular. The gelatin is rapidly liquefied. Gelatin stab cultures grow rapidly producing a funnel-shaped liquefaction process.

Bouillon. In bouillon the organism grows rapidly producing marked

cloudiness which is followed by the formation of a heavy, grayish-white pellicle.

Litmus Milk. Litmus milk is not acidified or coagulated.

Potato. The organism develops as a yellowish-brown growth on potato.

Peptone Solution. Vibrio metchnikovi produces indol in Dunham's peptone solution.

Nitrate Solution. The organism is capable of bringing about a reduction of nitrates to nitrites.

Pathogenicity. Vibrio metchnikovi can be readily differentiated from the vibrio of human cholera through the inoculation of guinea pigs. These animals when inoculated subcutaneously with Vibrio metchnikovi rapidly succumb, usually within 24 hours. The vibrio of human cholera injected in such manner is never rapidly fatal.

CHAPTER XIV

Order—Eubacteriales (Continued), Family—Bacteriaceae, Tribe—Chromobacterieae. Organisms of the Genus Pseudomonas

The family *Bacteriaceae* is one of the most important of the whole classification. It includes a large percentage of the organisms of importance both in human and veterinary medicine.

There are eleven tribes making up this family. They are: I—Chromobacterieae; II—Cellumonadeae; III—Achromobacterieae; IV—Lactobacilleae; V—Erwinieae; VI—Kurthieae; VII—Bacterieae; VIII—Pasteurelleae; IX—Klebsielleae; X—Hemophileae; and XI—Bacteroideae. From the standpoint of veterinary medicine we are interested only in four of them —Chromobacterieae, Bacterieae, Pasteurelleae and Hemophileae. The other seven tribes are at present made up of bacteria of no particular importance to the veterinarian.

The tribe Chromobacterieae is made up of water and soil bacteria which produce red, yellow, violet or blue-green pigment. It is divided into four genera but insofar as the purpose of this work goes but one genus (Pseudomonas) is of interest. The characteristics described for this genus by the Committee of the Society of American Bacteriologists are as follows:

> Principally water and soil bacteria producing a water-soluble pigment which diffuses through the medium as green, blue or yellowish-green. Motile or non-motile. Gram-negative.

PSEUDOMONAS AERUGINOSA (BACILLUS PYOCYANEUS, BACTERIUM PYOCYANEUM, ETC.)

Under the term "Pseudomonas aeruginosa" we now recognize the organism which has been commonly known as "Bacillus pyocyaneus," the causative factor of green or blue pus. This organism occurs as a rather widespread saprophyte being found in water, sewage, manure, etc. Further, it is a frequent inhabitant of the intestinal tracts of normal animals.

The pathogenic property of Pseudomonas aeruginosa was first noted by Gessard in 1882. He demonstrated that this organism was a factor in suppurative processes discharging greenish or bluish pus. While Pseudomonas aeruginosa is commonly found associated with other pyogenic organisms in suppurating processes, it is in itself pathogenic.

Morphology and Staining Characteristics. Pseudomonas aeruginosa is a rather short, slender rod, the average usually measuring 2 to 2.5 microns in length and 0.3 to 0.5 micron in thickness. There is quite a variation, however, in size, some cells appearing much larger than the average, others smaller. The organism is found singly, in pairs and rather frequently in short chains. It is actively motile, possessing a flagellum at one pole. It does not form spores. It stains well with the ordinary aniline dyes and is Gram-negative.

Cultural Characteristics. Pseudomonas aeruginosa grows readily upon

Fig. 27. Pseudomonas aeruginosa

the usual laboratory media. It is an aerobe and facultative anaerobe. Pigment production, however, does not take place when the organism is grown in the absence of oxygen.

Agar. On agar plates, rather large, irregular, grayish colonies with dark centers appear. The medium surrounding the colonies takes on a greenish hue. The growth on agar slants is moist and of a yellowish-green color, the green pigment soon penetrating the medium giving it a bright green hue which later becomes much darker.

Gelatin. Colonies on gelatin plates possess a rather dark center surrounded by a less dense, granular zone which radiates out to the periphery

forming an irregular border. The medium about the colonies has a fluorescent green color which becomes darker as the culture ages. The gelatin is usually completely liquefied within 4 or 5 days. In gelatin stab cultures, liquefaction starts at the surface forming a funnel-shaped area, the process gradually extends downward. A greenish-yellow color is produced in the uppermost portion of the culture.

Bouillon. Growth in broth is rapid with the production of considerable turbidity and pellicle formation. A yellowish-green color is imparted to the medium, particularly in the upper stratum of the fluid. As the culture ages sedimentation occurs.

Litmus Milk. Litmus milk is coagulated and acidified by Pseudomonas aeruginosa. The medium subsequently assumes a yellowish-green color.

Potato. The organism grows well on potato producing a brownish pigment with dark green discoloration of the medium.

Peptone Solution. Small amounts of indol are produced in Dunham's peptone solution.

The Pigments of Pseudomonas aeruginosa. Pseudomonas aeruginosa possesses two pigments, one a fluorescent green which is water soluble and the other a bluish-green, soluble in chloroform. The bluish-green pigment is known as "pyocyanin" and is characteristic of Pseudomonas aeruginosa. It can be extracted from cultures with chloroform and crystallized out in the form of blue crystals. The water-soluble, fluorescent green pigment is common to a variety of bacteria.

The base for the pyocyanin, in cultures grown in the absence of oxygen, is a colorless product which assumes its characteristic hue only after oxidation. Pyocyanin is rendered colorless in the presence of active reducing agents.

Protein Ferment. Pseudomonas aeruginosa produces a type ferment known as "pyocyanase" which is capable of splitting albumin and also certain bacteria. This ferment is exceedingly thermostable. Experiments have been conducted in which animals were inoculated with mixtures of pyocyanase and anthrax bacilli. The disease fails to develop in a number of such instances due to the disintegration of the bacteria by the pyocyanase.

Toxin Production. In addition to its pigments and ferment, Pseudomonas aeruginosa produces an endotoxin and a feeble soluble toxin against which an antitoxin can be prepared.

Resistance. Pseudomonas aeruginosa will resist drying for several weeks when contained in pus or exudates. It is destroyed in one-half hour at a temperature of 60°C. Boiling destroys it immediately. It succumbs to the action of all of the common disinfectants in the usual strength solutions.

CHAPTER XV

Order—Eubacteriales, Family—Bacteriaceae (Continued), Tribe—Bacterieae. Organisms of the Genus Escherichia

The tribe "Bacterieae" is composed of Gram-negative rods which usually grow well on artificial media. They generally attack carbohydrates, producing acid and frequently gas. Motile forms have peritrichous flagella. There are six genera making up this tribe—"Escherichia," "Aerobacter," "Proteus," "Salmonella," "Eberthella" and "Alcaligines."

In dealing with bacteria of the intestinal tract we have the *colon* or *Escherichia organisms* at one end of the group and at the other end *Eberthella typhi* (typhoid bacillus). Intermediate between these we have the *paratyphoid bacteria*, sometimes referred to as organisms of the *intermediate group* and under our present nomenclature as the *Salmonella group*. Under the genus Escherichia we consider the "colon" organisms.

In 1886 Escherich[1] demonstrated in the feces of normal, milk-fed infants an organism which he designated "Bacterium coli commune." This finding marked the beginning of an enormous amount of work with bacteria of the intestinal tracts of man and the lower animals. Out of this work has come our present knowledge of the now well-known *colon-paratyphoid-typhoid group* of bacteria.

Among the inhabitants of the intestinal tract of man and the lower animals are several varieties of closely-related bacteria which are commonly termed "colon bacilli." These organisms, when confined to the intestinal tract, are not only non-pathogenic but from all indications actually subserve a useful purpose in that they probably aid in the splitting of carbohydrates. Further, their presence in the intestinal tract is antagonistic to certain forms of putrefactive bacteria.

The characteristics of this genus as given by the Committee of the Society of American Bacteriologists follow:

> Motile or non-motile rods, commonly occurring in the intestinal canal of normal animals. Attack numerous carbohydrates forming acid and frequently acid and gas. Do not produce acetyl-methyl-carbinol.

The following species are considered in this work: Escherichia coli, Escherichia communior, and Escherichia acidi-lactici.

[1] Escherich, Die Darmbakterien des Sauglings, Stuttgart, 1886.

ESCHERICHIA COLI (BACILLUS COLI COMMUNIS)

This organism is the type species of the genus. It is the most common of the group and has been the subject of a vast amount of study. It is common in the intestinal tract of the various domestic animals but occurs in much smaller numbers in the intestines of the horse than in the cow, pig, sheep, etc.

Morphology and Staining Characteristics. Escherichia coli is a short, rather plump rod, the average measuring about 0.50 micron in width and

FIG. 28. ESCHERICHIA COLI

from 1 to 3 microns in length. The organism frequently appears almost coccus-like, especially when present in tissues or when grown under unfavorable conditions. It occurs singly, in pairs and occasionally is seen in short chains. It is motile by means of peritrichous flagella. The degree of motility, however, varies greatly with different strains and under different conditions. Some strains are very actively motile while others possess motility but little more marked than Brownian movement. As a general rule, young cultures of recently isolated strains are actively motile.

The organism stains well with the ordinary aniline dyes in aqueous solutions and is Gram-negative. Escherichia coli frequently stains bipolar, particularly when found in a pathogenic rôle in tissues or exudates.

Cultural Characteristics. Escherichia coli grows exceedingly well on most of the ordinary culture media. It is an aerobe and facultative anaerobe. It will propagate between rather wide temperature limits, multiplication taking place at temperatures ranging between 10° and 40°C. Its optimum temperature, however, is around 37.5°C. It is not very fastidious as to the reaction of the medium in which it is grown. It will grow readily between rather wide ranges of reaction.

Agar. Round, raised, grayish or slightly yellowish-white, moist colonies develop within 14 to 16 hours. These are slightly opaque in the beginning, becoming markedly so as the cultures age. On *agar slants* a uniform, moist, grayish or slightly yellowish-white growth develops.

Lactose-Litmus Agar. On this medium the colonies and area immediately surrounding them are red, due to acid resulting from fermentation of the lactose, the litmus turning red under such circumstances. As the culture becomes older the acidification extends until the entire plate has lost its blue hue and taken on the acid pink color.

Eosin-Methylene-Blue Agar. This medium is highly valuable for the identification of organisms of the colon group. Colonies of these organisms on this medium appear black or have black centers and commonly possess a metallic sheen. The eosin and methylene blue, through the protection afforded by the agar (which acts as a protective colloid), is not precipitated except when acid is produced. Colon bacilli fermenting the lactose in the "E.M.B." medium with the production of acid bring about a precipitation of the dyes which results in the black colonies described.

Russell's Double Sugar Medium. This is another medium of great value in differentiating colon organisms from those of the Salmonella and typhoid groups. Colon bacilli produce a large amount of gas in this medium and both the butt and slant is acidified, turning yellow where phenol red is the indicator used.

Bouillon. Growth in this medium is rapid with marked clouding and sedimentation. Pellicle formation occurs with some strains.

Endo Medium. Colon colonies on Endo medium appear red with a metallic sheen.

Gelatin. Colonies on gelatin plates appear opaque, grayish-white to yellowish, and finely granular, becoming more coarsely granular and darker as they age. Where the medium is firm the colonies possess rather smooth, well-defined borders. In soft medium the borders are apt to be irregular due to protruding growths from the colony. In gelatin stab cultures the growth spreads over the surface of the medium and is also well marked along the line of the stab. No liquefaction of the gelatin occurs.

Litmus Milk. Litmus milk is acidified and coagulated.

Potato. Escherichia coli grows well on potato. The growth at first is of a grayish-white color later becoming a yellowish-brown.

Peptone Solution. Indol is produced by the growth of Escherichia coli in Dunham's peptone solution.

Nitrate Solution. Nitrates are reduced to nitrites by this microörganism.

Fermentable Media. Escherichia coli produces acid and gas in broth containing dextrose, lactose, mannite, maltose, levulose, galactose, raffinose, arabinose, and dulcite. It does not ferment saccharose which fact readily distinguishes it from Escherichia communior.

Toxic Products. Escherichia coli does not secrete a soluble toxin but a very irritating and rather potent endotoxin can be demonstrated in extracts of the bacteria.

Pathogenicity. As indicated in the beginning Escherichia coli is ordinarily non-pathogenic when confined to the intestinal canal. However, it frequently assumes marked pathogenicity when it gains entrance to, or is introduced in, other parts of the body. Pyosepticemia (joint ill, navel ill) and dysenteria neonatorum (white scours) of sucklings are frequently due to virulent strains of Escherichia coli. Cystitis, pyonephrosis, cholecystitis, fistulae, peritonitis and abscesses, and even septicemia have at times been found to be due to the activities of this microörganism.

Resistance. Escherichia coli is not a very resistant organism. Exposure to a temperature of 62°C. for 10 minutes destroys it. Desiccation kills it in a very short time. Phenol, bichloride of mercury, liquor cresolis compound, formalin, etc., in the usually employed dilutions, all are effective in destroying this organism.

Immunization. Fairly good results have been obtained in the prevention and treatment of pyosepticemia and dysenteria neonatorum, when caused by Escherichia coli, with an immune serum prepared by immunizing horses or cattle to a variety of strains of the organism isolated from cases of such ailments. Considerable immunity can also be produced by the use of killed cultures of the organism.

ESCHERICHIA COMMUNIOR (BACILLUS COLI COMMUNIOR)

Escherichia communior in its morphology and staining characteristics is similar in every way to Escherichia coli. In culture it differs from the latter species in that it produces acid and gas in saccharose bouillon.

Escherichia communior was given the name "communior" because when first identified it was believed to be more common in the intestinal tract than the communis variety. This belief has since been shown to be erroneous.

ESCHERICHIA ACIDI-LACTICI (BACTERIUM ACIDI-LACTICI)

Escherichia acidi-lactici is a non-pathogenic organism but is mentioned here because it is frequently found in milk, cheese, water and in the intestinal tract of man and animals. It is very similar to Escherichia coli but may be distinguished by the fact that it does not ferment dulcite. Further, it is non-motile but as some cultures of Escherichia coli are only very slightly motile this factor is only of definite differentiating value when distinct motility is observed, in which case it can, of course, be concluded that the organism under consideration is not Escherichia acidi-lactici.

ORDER—EUBACTERIALES, FAMILY—BACTERIACEAE, TRIBE—BACTERIEAE (CONTINUED). ORGANISMS OF THE GENUS AEROBACTER

At the present time there are no pathogenic species of this genus of importance in veterinary medicine. However, as one species, Aerobacter aerogenes, is very similar to organisms of the genus Escherichia and is commonly found in milk, water, feces, etc., it is merely mentioned here with the view of calling attention to it so that the student will not mistake it for a species of Escherichia. Further, this organism is an important factor in the natural souring of milk and in this respect is of interest to veterinarians.

The Committee of the Society of American Bacteriologists' specifications for the genus Aerobacter are:

Motile or non-motile rods, commonly occurring in the intestinal canal of normal animals. Produce acetyl-methyl-carbinol.

AEROBACTER AEROGENES (BACILLUS AEROGENES, BACTERIUM LACTIC AEROGENES)

As indicated above this organism in many ways is very similar to organisms of the Escherichia group. It is distinguished from same by the following characteristics: It ferments dextrose, lactose, and saccharose but not dulcite. Growth in bouillon containing such sugars thus serves to differentiate it from Escherichia coli, Escherichia communior, and Escherichia acidi-lactici. The fact that it does not ferment dulcite distinguishes it from the first two and its positive action on saccharose differentiates it from Escherichia acidi-lactici. It is non-motile. Very frequently the organism appears to have a distinct capsule. Voges-Proskauer test for acetyl-methyl-carbinol is positive.

Aerobacter aerogenes is but rarely pathogenic. It has occasionally been incriminated as the causative factor in cystitis.

CHAPTER XVI

Order—Eubacteriales, Family—Bacteriaceae, Tribe—Bacterieae (Continued). Organisms of the Genus Salmonella

This genus consists of a number of Gram-negative, dextrose-splitting organisms making up the so-called "paratyphoid," "enteritidis," "intermediate," or "hog cholera" group which occupies a position intermediate between the colon and typhoid organisms. The genus was first designated "Salmonella" by Ligineres, in honor of the late Dr. D. E. Salmon, formerly Chief of the United States Bureau of Animal Industry, in recognition of his work on hog cholera.

This group includes the organisms responsible for paratyphoid fever and food-infections ("food-poisoning") in man, and in the lower animals, equine infectious abortion, bacillary white diarrhea of chicks, mouse typhoid, paratyphoid infections, various secondary conditions, etc.

There are two types of organisms making up this group. The organism formerly designated "Bacillus paratyphosus A" is now termed "Salmonella paratyphi." It is by far the least common. The organism formerly spoken of as "Bacillus paratyphosus B," is now referred to as "Salmonella schottmülleri." The various other members known formerly as "Bacillus enteritidis," "Bacillus typhi-murium," "Bacillus suipestifer," "Bacillus abortus-equi," "Bacterium anatum," etc., still retain their specific names but are given the new generic name "Salmonella." These latter organisms are practically all almost indistinguishable from the "B" type or Salmonella schottmülleri.

The characteristics of the genus as specified by the Committee of the Society of American Bacteriologists follow:

Motile forms. Generally occur in the intestinal canal of animals, in various types of acute, inflammatory conditions. Attack numerous carbohydrates with the formation of both acid and gas. In general do not form acetyl-methyl-carbinol.

salmonella schottmülleri (bacillus paratyphosus "b")

Salmonella schottmülleri is the type species of the Salmonella genus. It is the cause of paratyphoid fever in man and, further, is also capable of producing food infection ("food-poisoning") in man ingesting meat, meat-food, dairy products, or other food-stuffs contaminated with the organism.

When present in animals Salmonella schottmülleri is most frequently found in the intestinal tract although it is occasionally found in the udder of cows. It is, however, not nearly so common in the lower animals as the Salmonella enteritidis, Salmonella suipestifer, Salmonella aertrycke, Salmonella typhimurium, Salmonella abortus-equi, etc.

Some investigators are of the opinion that the type of Salmonella schottmülleri which produces paratyphoid fever in man is a strain from human source, whereas that producing food infection is probably of animal origin. While there is a bare possibility that this may be true, at the present time,

FIG. 29. SALMONELLA SCHOTTMÜLLERI

we have no means of distinguishing between those types which may be of human origin and those from animals.

Morphology and Staining Characteristics. Microscopically Salmonella schottmülleri and others of the same genus are practically indistinguishable from Escherichia coli and the typhoid fever organism. The rods measure from 1.5 to 2.0 microns in length and from 0.5 to 0.8 micron in width. They occur singly or in pairs and are motile by means of peritrichous flagella. It stains well with the various aniline dyes and is Gram-negative.

Cultural Characteristics. Salmonella schottmülleri is an aerobe and facultative anaerobe. It grows well on media of slightly acid or alkaline reaction.

It propagates best at the usual incubator temperature but will grow at considerably lower temperatures.

Agar. Colonies on agar plates are circular, regular in outline, moist, and of a grayish or bluish-gray color. On slant agar the growth is fairly heavy, moist and of a grayish color.

Gelatin. The colonies on gelatin plates are similar to those on agar. In stab cultures growth occurs on the surface and along the stab. The medium is not liquefied.

Bouillon. Salmonella schottmülleri renders broth uniformly turbid with sedimentation and the formation of a light, gray pellicle.

Litmus Milk. This medium is at first rendered slightly acid but later it becomes alkaline.

Potato. The growth of Salmonella schottmülleri on potato is usually light and of a moist, grayish-white character.

Peptone Solution. Indol is not produced in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites by Salmonella schottmülleri.

Fermentable Media. Sugar fermentation tests constitute a simple and accurate means of readily differentiating this and other organisms of the genus from colon and typhoid organisms. Dextrose is fermented whereas lactose is not. Acid is produced in media containing rhamnose. These facts at once differentiate it from Escherichia coli and Escherichia communior which ferment lactose and fail to produce acid from rhamnose. On the other hand, the typhoid organism is readily differentiated in that it does not split either dextrose or lactose nor produce acid from rhamnose. Salmonella schottmülleri also produces acid and gas in broth containing galactose, levulose, maltose, mannite, dulcite and xylose. As xylose is not fermented by Salmonella paratyphi broth containing this agent is highly valuable in differentiating the two types of organisms.

Russell's Double Sugar Medium. Salmonella organisms can be readily distinguished from those of the Escherichia group through the use of Russell's double sugar medium. Stab inoculations in such medium result in only a small amount of gas, evidenced by a few gas bubbles in the depths of the agar, as only the small amount of dextrose in the medium is split. Further, the butt of the medium shows the acid yellow color (where phenol red is used as indicator) while the slant remains red.

Litmus Agar, "E.M.B." and Endo Media. These media are all very valuable in differentiating this organism from the Escherichia group. Salmonella schottmülleri not fermenting lactose fails to produce the characteris-

tic colonies on these media as described in connection with Escherichia coli.

Lead Acetate Agar. This medium is of value in differentiating Salmonella paratyphi and Salmonella schottmülleri. Stab cultures in lead acetate agar result in a blackening or brownish discoloration of the medium when Salmonella schottmülleri is inoculated. Salmonella paratyphi does not discolor the medium. Salmonella enteritidis and Salmonella typhi-murium turn the medium brown the same as Salmonella schottmülleri.

Serological Reactions. Agglutination tests are often of value but cannot always be relied upon to differentiate members of the Salmonella group because of cross agglutination. Agglutinin absorption tests are more valuable.

Resistance. Salmonella schottmülleri is destroyed by rather low temperatures. Moist heat of 58°C. will kill it within 20 minutes. It readily succumbs to the action of the ordinary antiseptics in the usual strength solutions.

Immunization. It is possible to immunize against Salmonella schottmülleri with properly prepared bacterins. In man the results from immunization against paratyphoid fever have been highly satisfactory.

SALMONELLA AERTRYCKE (BACILLUS AERTRYCKE, BACILLUS PESTIS CAVIAE)

Salmonella aertrycke is frequently responsible for outbreaks of disease among guinea pigs and in this respect has caused serious trouble in various laboratories and breeding establishments. This organism is also a frequent offender in food infections ("food-poisoning") in man. It occasionally occurs as an inhabitant of the intestinal tracts of hogs, cattle, fowls, etc.

Salmonella aertrycke is very similar to Salmonella schottmülleri in its morphology, staining and cultural characteristics, tenacity, etc. As a matter of fact, it is very difficult to distinguish them. Serologically, Salmonella aertrycke does not absorb all agglutinins in Salmonella schottmülleri immune serum.

SALMONELLA ENTERITIDIS (BACILLUS ENTERITIDIS, GÄRTNER BACILLUS)

From the standpoint of food infection in man Salmonella enteritidis is an old offender. As a matter of fact, this was the first member of the intermediate, paratyphoid, or Salmonella group, demonstrated to be a factor in "food-poisoning" in man. In 1888 Gärtner[1] isolated this organism from

[1] Gärtner, Correspond. d. Allgemein. Artzl. Vereins, Thuringen, 1888.

"emergency slaughtered" beef which had caused a severe gastro-intestinal disturbance in more than fifty individuals. The microbe was also isolated from tissues of a fatal case among the affected persons.

Salmonella enteritidis is capable of producing marked gastro-intestinal disturbances and even septicemia in lower animals. Animals which have recovered from such infections, and in some cases animals with no history of disease, are often found as carriers of the organism, harboring the same in their intestinal tracts. In addition milch cows are occasionally found harboring Salmonella enteritidis in their udders. The organism is commonly "carried" by rats and mice and infection of various food-stuffs is frequently accomplished by contamination with the feces of such carriers.

This organism is well-known for its toxic properties. It does not secret a true toxin but contains an endotoxin which is very resistant to heat, even tolerating prolonged boiling.

Salmonella enteritidis can be distinguished from Salmonella schottmülleri and the other closely related organisms of the group only with the greatest difficulty. Morphologically and culturally there are no definite, constant differences by which it can be readily identified. Where animal inoculation can be resorted to the toxic nature of killed cultures is of some value in differentiation. Agglutination tests can be resorted to but, as pointed out in the beginning, they cannot be considered entirely reliable because of degrees of cross reactions. Agglutinin absorption tests are more reliable.

SALMONELLA SUIPESTIFER (BACILLUS SUIPESTIFER, BACILLUS CHOLERA SUIS)

This is another member of the Salmonella group responsible for food infection in man. In the lower animals it is best known as a secondary factor in hog cholera. In 1885 Salmon and Smith[2] isolated this organism from pigs affected with cholera and believed it to be the cause of the disease. This was the first description published of an organism of the group which is now named in honor of Salmon. As is now well known, Salmonella suipestifer has been shown to be merely a secondary invader in hog cholera, the primary etiological factor being a filterable virus.

Salmonella suipestifer is a very common inhabitant of the intestinal tract of hogs. Under normal conditions it is of no significance but under conditions of lowered resistance and other primary infections it becomes of considerable importance as a secondary factor. It is thus a facultative pathogenic organism. In hog cholera it is found associated with other

[2] Salmon and Smith, Ann. Rpt., U. S. Bureau of Animal Industry, 1885.

bacteria in intestinal ulcers, in the various organs, particularly the spleen, and in the circulation.

Because of its widespread distribution in nature and its common inhabitation in the intestinal tract of lower animals, its opportunity to infect meat, meat-food and dairy products is great.

Salmonella suipestifer possesses in general the morphological, staining and cultural characteristics of the Salmonella schottmülleri type of the Salmonella group. It differs from Salmonella schottmülleri in that it does not darken lead acetate agar. This factor might cause it to be confused with Salmonella paratyphi but it differs from that organism in that it

FIG. 30. SALMONELLA SUIPESTIFER

ferments xylose. In tenacity it likewise corresponds to the other members of the genus, 56°C. destroying it in one hour; 58°C. in about 20 minutes. Phenol, bichloride of mercury, formalin, liquor cresolis compound, etc., in the usual dilutions, are all effective within 15 or 20 minutes.

SALMONELLA TYPHI-MURIUM (BACILLUS TYPHI-MURIUM, BACILLUS OF MOUSE TYPHOID)

In 1892 Loeffler[3] announced the isolation of the organism now termed "Salmonella typhi-murium" as the etiological factor in an epidemic disease

[3] Loeffler, Centralblatt f. Bakteriologie, xi, 129, 1892.

of mice. Whether or not man and domestic animals are susceptible to this organism is a rather unsettled question. The evidence at hand, however, tends to indicate that Salmonella typhi-murium is capable of producing severe gastro-intestinal disturbances in man consuming food contaminated with the organism. Domestic animals are apparently seldom affected by Salmonella typhi-murium. This organism, however, frequently causes serious trouble for investigators or others interested in the raising of mice. White or gray mice fed the organism will succumb in 9 or 10 days. When administered subcutaneously to such animals it usually produces death within 48 hours.

Salmonella typhi-murium corresponds in its morphological, staining and cultural characteristics to Salmonella schottmülleri.

SALMONELLA PSITTACOSIS (BACILLUS PSITTACOSIS, BACTERIUM PSITTACOSIS)

Nocard[4] was the first to describe Salmonella psittacosis as the causative agent of a serious disease of parrots. Sick birds are apparently capable of transmitting the disease to man, the affection in the human family occurring as a severe and sometimes fatal pneumonia. Affected parrots develop a diarrhea, become weak, and are later prostrated with evidence of pulmonary involvement. Postmortem examination reveals small, grayish nodules in the liver, spleen, lungs, etc.

Like the preceding organism Salmonella psittacosis possesses the various morphological, staining and cultural properties of the other organisms of the Salmonella schottmülleri group.

SALMONELLA ABORTUS-EQUI (BACILLUS ABORTUS-EQUI, BACTERIUM ABORTUS-EQUI, BACTERIUM ABORTI-EQUI, BACILLUS ABORTIVO-EQUINUS)

From the veterinarian's standpoint this is an important member of the Salmonella group. While equine infectious abortion, due to Salmonella abortus-equi, is fortunately not so prevalent as bovine infectious abortion, in certain sections of the United States the disease has caused great losses.

The question of infectious abortion in both horses and cattle has been the subject of a vast amount of investigation, not only in the United States but in different parts of the world. A few investigators have been inclined to discount the importance of infectious abortion among animals of the equine, bovine and porcine species. While there are, obviously, other con-

[4] Nocard, Conseil d. Hyg. Publique et Salubrite du Dept. du Seine, Seance, March, 1893.

ditions and factors, including various microörganisms, capable of preventing conception and of causing abortion, the fact remains that in horses and in cattle there is an organism, in the case of each species, capable of being spread from animal to animal, which is preëminently an abortion-producing agent. In the case of the equine species the organism is Salmonella abortus-equi. In cattle it is Alcaligines abortus (Bang). The latter organism will be discussed in a subsequent chapter.

Morphology and Cultural Characteristics. Salmonella abortus-equi, in general, corresponds to the Schottmülleri type of organism. It differs, however, in the appearance of its colonies and growth on gelatin and agar. When grown on either of these media, especially when the same is somewhat

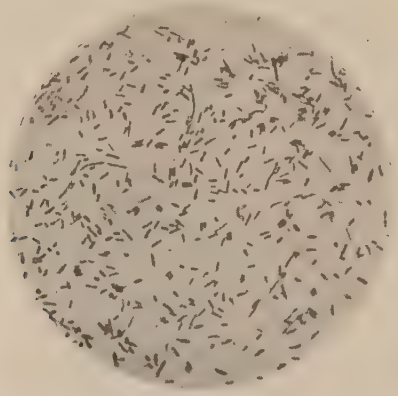


Fig. 31. Salmonella abortus-equi

dry, the growth in 48 to 72 hours takes on a characteristic dry, wrinkled or parchment-like appearance. On plate cultures it will be found that the colonies are not at all adherent to the medium and can be removed without material distortion by lightly pushing them with a platinum loop applied at a point on the edge of the colony. This characteristic wrinkled appearance is, in many instances, noted with the originally isolated culture. In some few cases, however, it does not become well-marked until after several generations on artificial media. In rare instances it fails to become well-marked.

Endotoxin. Salmonella abortus-equi possesses a rather potent endotoxin. Horses given subcutaneous injections of killed cultures or bacterins frequently develop a rather severe reaction, especially where large amounts are

administered. This is usually characterized by chills, fever, stiffness, inappetence, etc.

Resistance. This organism does not differ materially from Salmonella schottmülleri as regards its susceptibility to heat, chemical disinfectants, etc.

Serological Reactions. Agglutinins and complement-fixing bodies can be demonstrated in the blood of affected animals. The agglutination and complement-fixation tests, however, are not invariably positive immediately following an abortion due to Salmonella abortus-equi. A few days subsequent to the abortion, however, the test may be positive. This can probably be accounted for by the fact that the infection in mares is generally of an acute nature and with the process localized in the uterus, involving the fetal membranes and uterine wall, the abortion may occur before agglutinins and complement-fixing bodies have been produced in sufficient quantities to be demonstrable in the circulating blood.

Prophylaxis. Highly satisfactory results have been obtained with bacterins in the prevention of equine infectious abortion. The writer prepared such a bacterin for use at Army remount depots following a severe outbreak of equine infectious abortion and obtained excellent results from same.

For prophylactic purposes two series of three injections of bacterin are administered to mares in the fourth and ninth months of pregnancy. While reactions frequently follow the injection of the bacterin, it practically never results in abortion.

The technic of the agglutination and complement-fixation test for equine infectious abortion is given in the section on Serology. For the production of bacterins see section on the Preparation of Veterinary Biological Products.

SALMONELLA ANATUM (BACTERIUM ANATUM)

In the spring of 1918 Rettger[5] investigated a disease referred to as "keel" among young ducklings in Connecticut and isolated an organism of the colon-typhoid group which was subsequently shown to be the cause of the malady. The work with the etiological agent was carried out by Rettger and Scoville[6] and the organism designated by them as "Bacterium anatum."

The disease is highly fatal and is characterized by weakness, depression, chilliness, marked thirst and staggering. It is noted that some of the sick birds, after drinking, will draw themselves up to full height, stagger for a few seconds, keel over and die. Thus, the disease has been popularly termed "keel."

[5] Rettger, Abs. Bact., viii, 1919.

[6] Rettger and Scoville, Jour. Inf. Dis., xxvi, 217, 1920.

Postmortem examinations of ducklings dead of the disease reveal no characteristic macroscopic changes. As a matter of fact, aside from possible paleness of the tissues generally, no changes are usually discernible. However, Salmonella anatum can be readily isolated from the liver, spleen, lungs and heart blood of birds dead from the disease.

There is evidence which tends to indicate that the infection is transmitted from infected ducks to the ducklings through the eggs in a manner similar to that noted in white diarrhea of chicks.

Morphology and Staining Characteristics. Salmonella anatum is a short, actively motile, non-sporulating rod with rounded ends. The average organism measures 1 to 2 microns in length and about 0.50 micron in thickness. It usually occurs singly. It stains well with the ordinary aniline dyes and is Gram-negative.

Cultural Characteristics. This organism is an aerobe and facultative anaerobe with an optimum temperature requirement of 37.5°C. It will, however, propagate at room temperature.

Agar. There is nothing characteristic about the colonies of this microbe. On agar, moist, grayish-white colonies similar to those of other members of this group of organisms, develop within 24 hours. After 48 to 72 hours the growth is heavy and more opaque. The growth near the base of the medium in older cultures frequently appears somewhat wrinkled.

Gelatin. The colonies on gelatin plates are similar to those on agar. In stab cultures a scanty growth occurs along the line of the stab. The medium is not liquefied.

Bouillon. Broth is rendered distinctly turbid within 24 to 36 hours with the formation of a light pellicle.

Litmus Milk. During the first 48 hours litmus milk is rendered slightly acid. It then starts to turn alkaline, becoming markedly so in 12 to 14 days. No coagulation occurs.

Potato. The growth on potato is variable. Some strains produce a scanty growth on this medium while others grow luxuriantly on it. With some strains the growth, after several days, changes from a grayish to a light-brown color.

Fermentable Media. Salmonella anatum produces acid and gas from glucose, levulose, galactose, maltose, dextrin, arabinose, xylose, mannitol, dulcitol and rhamnose. Lactose, saccharose, inulin, raffinose and salicin are not productive of acid or gas.

Peptone Solution. Indol is not produced in Dunham's peptone solution.

Nitrate Solution. Nitrates are reduced to nitrites.

Pathogenicity. This organism is not pathogenic for rabbits, guinea pigs and mice. Young chicks, however, can be successfully infected.

Resistance. In tenacity this organism corresponds to the other members of the Escherichia-Salmonella-Eberthella group.

Serological Reactions. Agglutination reactions in dilutions as high as 1:4000 or more can be obtained with suspensions of Salmonella anatum and serum from rabbits immunized against the organism. A certain amount of cross agglutination can be demonstrated between Salmonella anatum and Salmonella schottmülleri.

Biological Therapy and Prophylaxis. Apparently no work has been done with biological products in the treatment or prevention of "keel."

SALMONELLA PULLORUM (BACILLUS PULLORUM, BACTERIUM PULLORUM)

Salmonella pullorum is responsible for white diarrhea of chicks, one of the greatest scourges of poultry husbandry. The organism was first described by Rettger and Harvey[7] in 1908. The disease attacks young chicks in the first few days of life and results in a very high mortality.

The organism is frequently carried in the ovaries of hens. While a large percentage of ova of hens with such ovaries never mature, such birds lay some fully developed eggs, capable of hatching, which contain the organism. The resulting chick, in such cases, has white diarrhea and readily infects others in the brood which may have been hatched from eggs free of the organism.

A large percentage of carriers result from those females which survive the disease. According to Rettger, Kirkpatrick and Jones[8] over 25 per cent of recovered females become permanent carriers.

Morphology and Cultural Characteristics. Salmonella pullorum possesses, in the main, the characteristics of the type species (Salmonella schottmülleri) of the group but in several important respects it differs. First of all, it is non-motile. Then, some strains, at times, will cease to produce gas from dextrose. The same strain on subsequent cultivation ordinarily regains its ability to split dextrose. This indifference towards dextrose is not characteristic of the typical Salmonella organism which is preëminently a dextrose fermenter. Salmonella pullorum does not split maltose, differing from the typical species in this respect. Dulcite and dextrin are likewise not split. In its staining characteristics and tenacity Salmonella pullorum does not differ from the other members of the group.

[7] Rettger and Harvey, Jour. Med. Res., xviii, 1908.

[8] Rettger, Kirkpatrick and Jones, Bul. No. 7, Storrs Agric. Exp. Sta., 1914.

Serological Reactions. In combatting white diarrhea of chicks the detection of hens with affected ovaries is of great importance. In this connection Jones[9] demonstrated that the macroscopic agglutination test can be used to good advantage. Serum from suspected birds agglutinating in dilutions of 1 to 100 or greater should be considered positive.

Immunization. Because of the fact that the disease attacks young chicks during the first few days of life, attempts at immunization, obviously, are out of the question.

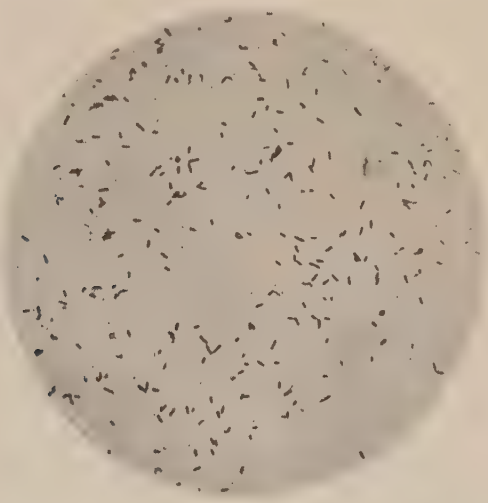


FIG. 32. SALMONELLA PULLORUM

SALMONELLA GALLINARUM (BACILLUS GALLINARUM, BACTERIUM GALLINARUM, BACTERIUM SANGUINARIUM)

Lemaistre,[10] writing in 1864, was probably the first to refer to an epidemic disease of poultry as "typhus" or "typhoid." Klein,[11] however, was the first to name the causative agent of fowl typhoid. His publication in 1889 referred to the etiological factor as "Bacillus gallinarum."

[9] Jones, Rpt. N. Y. State Vet. Col., 1911–12, p. 149.

[10] Lemaistre, Rec. Med. Vet., 1873, p. 376.

[11] Klein, E., Neber eine epidemische Krankheit der Hühner verursacht durch eine Bacillus—Bacillus gallinarium. Centbl. f. Bakt., Parasit. und Infektionskrankheiten, vol. 21, 1889.

In 1895 Moore[12] described, under the name of "Bacterium sanguinarium," an organism from cases of what he termed "infectious leukemia" of fowls. This organism was subsequently shown to be identical with Klein's Bacillus gallinarum. Apparently, the pronounced leukemia noted by Moore in the malady he investigated happened to be an outstanding manifestation in what was actually an outbreak of fowl typhoid. Under the present classification the organism is designated "Salmonella gallinarum." It differs, however, in several respects from the type species of this genus.

Morphology and Staining Characteristics. Salmonella gallinarum is a small rod with round ends, measuring from 0.3 to 0.50 micron in breadth and from 0.80 to 1.5 microns in length. Some forms appear as short ovals. It occurs singly and in pairs. It differs from the typical Salmonella organisms in that it is non-motile. The organism stains with the ordinary aniline dyes and is Gram-negative. It frequently shows "peripheral staining" leaving the central portion unstained. This peripheral staining differs from polar staining in that the organism is stained not only at the poles but shows border staining between the same. The organism is present in the intestinal contents, in the blood and in the internal organs.

Cultural Characteristics. Salmonella gallinarum is an aerobe and facultative anaerobe. Its optimum temperature requirement is 37°C. It grows well on media of slightly acid or alkaline reaction. If there is a choice, slightly alkaline media are possibly most conducive to growth.

Agar. On agar plates, small, grayish, glistening colonies develop in 24 hours. The growth on agar slants is moderate in amount and of a moist, grayish, glistening character.

Gelatin. Colonies on gelatin are similar to those on agar. There is no liquefaction.

Bouillon. The organism grows well in broth causing a uniform clouding with subsequent sedimentation. Some cultures produce thin pellicles.

Litmus Milk. Litmus milk is rendered alkaline although this may be preceded by evidence of slight acidity.

Potato. The organism grows well on potato, producing a yellowish, glistening growth.

Peptone Solution. Indol is not produced in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites.

Fermentable Media. Acid is produced from dextrose, maltose, dulcite, xylose, galactose, and levulose.

[12] Moore, Report of the B. A. I., U. S. Dept. of Agric. (12th and 13th).

Endotoxins. Some strains of Salmonella gallinarum possess a potent endotoxin as filtrates of old cultures are frequently found highly toxic.

Resistance. Salmonella gallinarum is similar to other members of the genus as regards resistance.

Serological Reactions. Agglutinins can be demonstrated in the blood serum of affected birds. Serum from such birds will frequently agglutinate antigen prepared from Salmonella gallinarum in rather high dilutions (1:1000).

Immunization. Results from attempted immunization of birds with killed or attenuated cultures have been discouraging.

SALMONELLA PARATYPHI (BACILLUS PARATYPHOID "A")

From a veterinary standpoint Salmonella paratyphi is but of little importance. The organism is of importance in human medicine because of its rôle in paratyphoid fever. It is but seldom met with in the lower animals.

Morphology and Cultural Characteristics. Salmonella paratyphi is similar to Salmonella schottmülleri in morphology and staining characteristics. Culturally it differs from the type species in that it does not attack xylose. Further, lead acetate agar is not darkened by this organism. In other respects the organism is similar to Salmonella schottmülleri.

CHAPTER XVII

Order—Eubacteriales, Family—Bacteriaceae, Tribe—Bacterieae, (Continued). Organisms of the Genus Eberthella

The Committee of the Society of American Bacteriologists specifications for this genus are:

Motile or non-motile rods, generally occurring in the intestinal canal of man, usually in different forms of enteric inflammation. Attack a number of carbohydrates with the formation of acid but no gas. Do not form acetyl-methyl-carbinol.

The important species of this genus is Eberthella typhi the causative factor of typhoid fever of man. Such species is of interest to veterinarians only in that it occupies a key position in the colon-paratyphoid-typhoid group of bacteria, being given considerable mention in differential considerations. There are, however, several other species in this genus which are of concern in veterinary medicine.

From time to time various types of bacteria have been isolated and described in connection with typhoid-like and cholera-like diseases. In this connection there has been considerable confusion in differentiating some of these maladies and their etiological agents. Hadley[1] conducted a series of important studies which have been of great value in bringing about a better understanding of some of these maladies, yet a perusal of the literature, at the present time, will still reveal considerable disagreement.

At the present time, true fowl cholera or hemorrhagic septicemia of fowls is definitely known to be due to Pasteurella avicida, an organism described under the genus "Pasteurella." Further, true fowl typhoid is known to be caused by the organism first described by Klein as "Bacillus gallinarum" and classified and described in this work as "Salmonella gallinarum."

In addition to the true fowl typhoid organism, there are several other rather closely related bacteria which, while capable of primary pathogenicity under various conditions, are probably also secondary factors. Among such latter organisms are three described by Hadley as "Bacterium jeffersonii," "Bacterium pfaffi," and Bacterium rettgeri" respectively. These fall in the genus "Eberthella" and are briefly described below.

[1] Hadley, Philip, Rhode Island Agric. Exp. Sta. Bul. No. 174, May, 1918.

EBERTHELLA JEFFERSONII (BACTERIUM JEFFERSONII)

Eberthella jeffersonii is one of three organisms which Hadley[2] described in connection with his investigation of cholera and typhoid-like diseases of chickens. He isolated this organism from a severe cholera-like epidemic of poultry in Massachusetts in 1909 in which thousands of birds died. Autopsy of fowls succumbing to the infection shows congestion or pneumonic changes in the lungs, liver congested and frequently containing hemorrhages, spleen enlarged and softened and a marked enteritis. The organism was readily isolated from the intestinal tract, blood and internal organs of infected birds.

Morphology and Staining Characteristics. Eberthella jeffersonii is a short, non-motile, non-sporulating rod with rounded ends. It averages about 0.80 to 1.00 micron in length and 0.30 to 0.50 micron in thickness. It occurs singly and in pairs. The organism stains well with the usual aniline dyes. It does not show peripheral staining as noted with the fowl typhoid organism. Stained by Gram's method the organism is decolorized.

Cultural Characteristics. Eberthella jeffersonii is an aerobe and facultative anaerobe growing well at the usual incubator temperature of 37.5°C.

Agar. On agar plates the colonies appear rather small, are translucent and of a grayish color. On agar slants a moist, filiform, grayish growth occurs.

Gelatin. The colonies on gelatin plates are similar to those on agar. No liquefaction occurs.

Bouillon. The growth of this organism in broth is somewhat characteristic. In place of a well-distributed growth, such as occurs with the fowl typhoid organism, a flocculent growth occurs. The flocculi rapidly settle to the bottom of the tube leaving the supernatent broth clear.

Litmus Milk. Litmus milk is rendered alkaline, becoming translucent after a considerable time.

Potato. A rather heavy, grayish growth occurs on potato.

Peptone Solution. Eberthella jeffersonii does not produce indol in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites.

Fermentable Media. Eberthella jeffersonii produces acid in media containing the following substances: Dextrose, mannose, galactose, levulose, xylose, arabinose, maltose, dextrin, dulcite and mannite. No gas is produced.

Pathogenicity. Fowls fed cultures of the organism or inoculated subcu-

[2] Hadley, Rhode Island Agric. Exp. Sta. Bul. No. 174, May, 1918.

taneously or intramuscularly succumb in from 5 to 10 days. Culture administered to guinea pigs intraperitoneally produces death. Subcutaneous injections result only in a localized process.

Resistance. Eberthella jeffersonii is quite similar to the fowl typhoid organism as regards its resistance to heat and chemical agents. A temperature of 58° to 60°C. will destroy it within 20 minutes. The ordinary disinfectants in the usual strength solutions are effective.

EBERTHELLA PFAFFI (BACTERIUM PFAFFI)

This organism was isolated by Pfaff in 1904 from diseased canaries. Hadley[3] subsequently studied the organism, recognized it as a new species and named it "Bacterium pfaffi." Under our present system of classification it is included in the Eberthella genus.

Morphological and Cultural Characteristics. Eberthella pfaffi is a nonmotile rod averaging 1.4 microns in length and about 0.4 micron in thickness. It stains well with the ordinary aniline dyes without evidence of peripheral or bipolar staining. It is Gram-negative.

Cultural Characteristics. This organism is an aerobe and facultative anaerobe. It grows well at the usual incubator temperature of 37.5°C. but will also propagate less rapidly at lower temperatures.

Agar. Colonies on agar plates are usually small, moist and of a grayish-white color.

Gelatin. Eberthella pfaffi does not liquefy gelatin.

Bouillon. Broth is rendered turbid with sedimentation and the formation of a light pellicle.

Litmus Milk. This medium is unchanged by the growth of Eberthella pfaffi.

Potato. A moderate, whitish growth occurs on potato.

Peptone Solution. Indol is not produced in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites.

Fermentable Media. These are of value in differentiating this organism from closely related species. Acid is produced in media containing dextrose, levulose, xylose, arabinose, maltose, dextrin, mannite and salcin. Saccharose is not fermented. No gas is produced.

Pathogenicity. This organism is only slightly pathogenic for laboratory animals.

Resistance. Eberthella pfaffi is similar to Eberthella jeffersonii in its resistance to heat and chemical agents.

[3] Hadley, Rhode Island Agric. Exp. Sta. Bul. No. 174, May, 1918.

EBERTHELLA RETTGERI (BACTERIUM RETTGERI)

This organism was isolated in 1909 by Rettger in an investigation of a fowl epidemic in Connecticut. Hadley[4] working with the organism at a later date identified it as a distinct species and named it "Bacterium rettgeri." Under our present terminology it is termed "Eberthella rettgeri."

Morphology and Staining Characteristics. This organism occurs as a short, non-motile rod measuring from 0.50 to 0.80 micron in length. It stains satisfactorily with the common aniline dyes without evidence of bipolar or peripheral staining. It is Gram-negative.

Cultural Characteristics. Eberthella rettgeri is an aerobe and facultative anaerobe. It grows best at a temperature of 37.5°C. but will also propagate at lower temperatures.

Agar. The colonies on agar are not distinctive. They are small, homogeneous and of a grayish-white color.

Gelatin. Colonies on gelatin are similar to those on agar. This medium is not liquefied.

Litmus Milk. This medium is rendered alkaline in 8 days becoming translucent but is not changed in consistency.

Potato. A luxuriant, grayish-white growth occurs on potato.

Peptone Solution. Indol is not formed in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites.

Fermentable Media. The reaction in these media differentiate Eberthella rettgeri from closely related organisms. Media containing dextrose, levulose, galactose, mannose, xylose, adonite, mannite and salicin, are rendered acid. No acid is produced in media containing arabinose, raffinose, saccharose, lactose, maltose, dextrin, inulin, dulcite and erythrite.

Pathogenicity. The organism is pathogenic for fowls artificially inoculated.

Resistance. Same as preceding organism.

[4] Hadley, Rhode Island Agric. Exp. Sta. Bul. No. 174, May, 1918.

CHAPTER XVIII

Order—Eubacteriales, Family—Bacteriaceae, Tribe—Bacterieae (Continued). Organisms of the Genus Alcaligines

The genus Alcaligines contains several species of considerable importance in veterinary medicine. These are Alcaligines abortus, the causative factor of bovine infectious abortion, Alcaligines melitensis, the etiological agent in Malta fever of man and animals, and Alcaligines bronchisepticus, an important secondary factor in canine distemper.

The genus is divided into two major groups, one group including those organisms which liquefy gelatin, the other group being composed of those which do not liquefy gelatin. The three species of interest in veterinary medicine do not liquefy gelatin. The specifications for this genus, as given by the Committee of the Society of American Bacteriologists, are:

Motile or non-motile rods, generally occurring in the intestinal canal of normal animals. Do not form acetyl-methyl-carbinol. Do not ferment any of the carbohydrates.

Alcaligines abortus (Bang) (Bacterium abortus, Bacillus abortus, Brucella abortus)

One of the most important diseases of livestock, from an economic standpoint at least, is bovine infectious abortion. This malady strikes at the very source of the cattle industry. It is widespread throughout the United States and in various other countries.

While the contagious nature of bovine infectious abortion was recognized as far back as the early part of the nineteenth century, it was not until 1897 that Bang[1] isolated the causative factor which we now term "Alcaligines abortus." The disease has been the subject of extensive investigation in various parts of the world and there can be no doubt as to its vast importance.

While Alcaligines abortus is preeminently a parasite of the bovine species, swine, and to a lesser extent, other animals, are susceptible to the infection. There is, at the present writing, no absolutely definite evidence that man is susceptible. However, as the organism is capable of producing serious organic lesions in the lower animals it is conceivable that under certain favor-

[1] Bang, Zeit. f. Tier. Med., 1, 1897, 241.

able conditions it might prove pathogenic for man. This possibility is given a little weight by the fact that man is very susceptible to the Malta fever organism which Evans[2] has shown is almost indistinguishable from Alcaligines abortus.

The organism has been isolated from tonsils of children and positive agglutination reactions have been obtained with blood specimens from children fed cow's milk. In the latter connection experimental evidence tends to indicate that such positive agglutination reactions are due to absorption of antibodies (agglutinins) from the ingested milk and not to the activity of Alcaligines abortus in the body of the individual.

Bovine infectious abortion presents a carrier problem of considerable importance. Evidence indicates that a rather large percentage of infected cows at some time harbor Alcaligines abortus in their udders.

Alcaligines abortus possesses a marked affinity for embryonic tissue, its natural habitat being the epithelium of the chorion. In infected cows it is, of course, present in the uterus. Following abortion or parturition the organism usually disappears from the uterus in a comparatively short time but frequently is found to have migrated to the udder where conditions are apparently more suitable for its existence than the non-pregnant uterus. In such cases it becomes established in the udder where it persists for periods varying from a few weeks to 6 or 7 years or longer and is eliminated with the milk. Its presence in the udder produces no appreciable change or disturbance in the gland.

Guinea pigs inoculated with Alcaligines abortus develop tubercle-like foci in the spleen, liver, lungs and kidneys. This fact must be kept in mind when making guinea pig inoculation tests for tuberculosis. When milk from cows suspected of having tuberculosis is inoculated into guinea pigs for the demonstration of Mycobacterium tuberculosis, care must be taken not to mistake lesions due to Alcaligines abortus for those of tuberculosis. This error would, of course, be likely only where conclusions are drawn from the macroscopic appearance of the lesions, the bacteriological examination readily revealing the true nature of the nodules.

While Alcaligines abortus is, obviously, an organism mainly peculiar to the female, it is, nevertheless, occasionally found in males. Bulls have been found to harbor the organism in their seminal vesicles. In some such cases no lesions of consequence are present while in other instances the organism is responsible for marked lesions. Buck, Creech and Ladson[3]

[2] Evans, Jour. Inf. Dis., 22, 1918.

[3] Buck, Creech and Ladson, Jour. Agric. Research, xvii, no. 5, August 15, 1919.

in an examination of 325 bulls, isolated Alcaligines abortus from the vesiculae seminales of four.

As already indicated, Alcaligines abortus has an affinity for embryonic tissues, being found in great numbers in the epithelium of the chorion. It is also present in the uterine mucosa and inflammatory exudate. The stomach of the aborted fetus is a favorable organ to culture from for the isolation of the organism.

Alcaligines abortus brings about abortion by producing an inflammation which results in the formation of an exudate between the chorion and uterine mucous membrane, thus causing a loosening and finally separation of the fetal from the maternal tissues. In some cases the immediate expulsion of the fetus does not occur, but, because of the marked interference with nutrition resulting from the disturbed relationship between fetal and maternal tissues, the fetus becomes mummified and may have to be forcibly removed.

There has been considerable controversy as to the natural mode of infection with Alcaligines abortus. While investigators generally are agreed on the fact that infection readily takes place through the alimentary canal as a result of ingesting contaminated food and water, the possibility of infection by way of the genital tract has been denied by a number of prominent workers, particularly Schroeder. In contending against the bull as a transmitter of the infection it is held that if copulation between a normal cow and an infected bull is permitted only on "neutral" ground transmission of the disease will be avoided. The view is taken that the infection will not take place through the genital tract but where semen dripping from the penis of the bull or leaking from the vagina of the cow contaminates pastures, food or water, animals will subsequently become infected through the alimentary tract.

The writer adheres to the opinion that while the most important path of infection is the alimentary canal, that, nevertheless, under certain conditions, especially in cases of slight injury during services, infection of cows by way of the genital route is possible.

Morphology and Staining Characteristics. Alcaligines abortus is a small, non-motile, non-sporulating, pleomorphic rod varying from 0.50 to 1.50 microns in length and from 0.40 to 0.60 micron in thickness. The short forms are very difficult to distinguish from cocci. They usually appear singly but are also found in pairs. The organism stains satisfactorily with the ordinary aniline dyes and is Gram-negative.

Cultural Characteristics. Alcaligines abortus is peculiar in its atmospheric requirements when first cultured on artificial media. It will not grow on

initial culture when subjected to usual atmospheric conditions but requires either partial anaerobiosis or an atmosphere which has been displaced to the extent of about 10 per cent with CO_2. Partial anaerobiosis can be obtained by incubating the cultures in closed jars in which cultures of Bacillus subtilis are placed. Another method is to place the cultures of Alcaligines abortus in a jar with a small alcohol lamp. The lamp is lighted and the lid of the jar put tightly in place and sealed. The oxygen consumed by the flame before it expires leaves an atmosphere favorable to the growth of Alcaligines abortus.

While the initial isolation of Alcaligines abortus is not without difficulty, after the organism has once been obtained in culture on artificial media, it

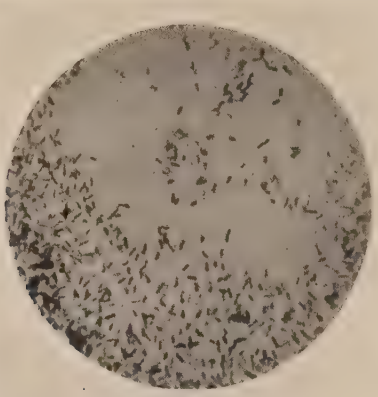


FIG. 33. ALCALIGINES ABORTUS

readily adapts itself to growth on same and subsequent subcultures can be grown under ordinary conditions without trouble. Alcaligines abortus has an optimum temperature requirement of about 37.5°C. but will grow less rapidly at lower temperatures. It will develop on media of either slightly acid or slightly alkaline reaction.

Agar. With initial cultures the growth on plain agar is very slow and is of a moist, glistening, opalescent nature, being very light. Cultures adapted to artificial media produce a heavy, slightly yellowish growth. On agar plates very small, slightly raised, opalescent, dewdrop-like colonies develop. Glycerin agar or glycerin-serum-agar is more preferable than plain agar.

Liver Infusion Agar (*Stafseth*). For the initial isolation of Alcaligines

abortus Huddleson[4] recommends a liver infusion agar prepared without excessive heating and filtered through glass wool rather than cotton or paper. The reaction is adjusted to pH 6.6 to 6.4 and one part of a saturated, aqueous solution of gentian-violet is added to 10,000 parts of the medium. The gentian-violet will inhibit a large percentage of bacteria other than Alcaligines abortus. He recommends incubation in air tight jars in which approximately 10 per cent of the air has been replaced by CO_2.

Gelatin. The colonies on gelatin are similar to those on agar. The medium is not liquefied.

Bouillon. Growth of the organism in bouillon produces a slight turbidity without pellicle formation. Glycerin bouillon or, better still, glycerin bouillon containing blood serum, is more favorable for the growth of Alcaligines abortus than plain bouillon.

Litmus Milk. Litmus milk is rendered slightly alkaline by Alcaligines abortus.

Potato. The growth of Alcaligines abortus on potato is somewhat like that of Pfeifferella mallei. A slight, brownish growth occurs after 48 hours, the medium subsequently taking on the brownish tinge.

Peptone Solution. Indol is not produced in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites.

Fermentable Media. Alcalignies abortus does not produce gas or acid in the fermentable media usually employed in bacteriological work.

Differentiation from Alcaligines melitensis. As previously indicated, Evans has called attention to the striking similarity between Alcaligines abortus and Alcaligines melitensis. Not only do they correspond closely in their morphological, staining and cultural characteristics, but they cross agglutinate. They can, to a certain extent, be differentiated by agglutinin absorption tests.

Further, Buck[5] has emphasized the point that in primary culture, the two organisms can be differentiated by the fact that Alcaligines melitensis can be cultivated on plate cultures under the ordinary atmospheric conditions, whereas Alcaligines abortus can only be initially cultivated under conditions of partial anaerobiosis or CO_2 replacement as already mentioned.

Resistance. Alcaligines abortus is very susceptible to heat and the usual disinfectants. A temperature of 58°C. destroys it in about 6 minutes. Phenol in 5 per cent solution, bichloride of mercury 1:1000, liquor cresolis compound, etc., bring about its destruction in several minutes.

[4] Huddleson, Mich. Agric. Exp. Sta. Tech. Bul. No. 49, November, 1920.

[5] Buck, Jour. Agric. Research, vol. xxix, no. 12, December 15, 1924.

In organs, fetal membranes, and uterine discharge which have become dry, the organism will remain alive for several months.

Serological Reactions. Animals infected with Alcaligines abortus will give reactions to the agglutination and complement-fixation tests. These tests are of value in detecting animals which are or have been infected with Alcaligines abortus. In the application of the agglutination test either blood serum or milk whey from the suspected animal or animals can be used. Blood serum, however, is preferable. In interpreting agglutination reactions the lowest dilution in which agglutination occurs which should be considered positive has been somewhat debated. Agglutination in a dilution of 1:50 or greater is considered by the United States Bureau of Animal Industry as an indication that an animal is infected, or was, at some time previously infected with Alcaligines abortus. In the case of cows harboring the organism in their udders, the agglutination test, according to Schroeder,[6] will be positive in dilutions of 1:200 or more.

Immunization. Cows which have aborted once may abort a second time but if they again conceive, a third abortion, as a result of Alcaligines abortus, is very rare. This tends to indicate that after natural infection considerable immunity is gained. However, sterility frequently results from the infection.

Methods aimed at artificial immunization have not been as satisfactory as hoped for. A vast amount of work has been done with biological products with the result that some claim success, others failure. At the present time the situation can be summarized by stating that little or no benefit has been derived from the use of bacterins (suspensions of killed bacteria), whereas with vaccines (suspensions of living organisms) the evidence is a little more favorable but still far from satisfactory. Further, the injection of heavy suspensions of living Alcaligines abortus into animals on a wholesale scale is open to criticism.

ALCALIGINES MELITENSIS (MICROCOCCUS MELITENSIS, BACTERIUM MELITENSIS, BRUCELLA MELITENSIS)

Alcaligines melitensis, the cause of "undulant," "Mediterranean," "Rock" or "Malta" fever in man and animals, was first isolated by Bruce in 1887 from a case of the disease occurring on the Island of Malta. The disease has been more or less endemic there for many years. It has not, however, been limited to this island as it has been reported from Italy, Greece, Turkey, Spain, India, Africa, France, the United States, etc. In

[6] Schroeder, A. V. M. A. Jour., vol. 13, no. 5, 1922.

the United States it has occurred sporadically along the Mexican border but within the last few years cases have been reported from several central western states.

The disease in man is characterized by headache, malaise, inappetence and high fever which continues intermittently for a number of weeks. Enlargement of the spleen and liver occurs. Secondary complications such as arthritis, neuritis, orchitis, etc., are not uncommon.

In animals the disease seldom assumes an acute nature. Affected goats and sheep develop only a moderate fever which may escape attention.

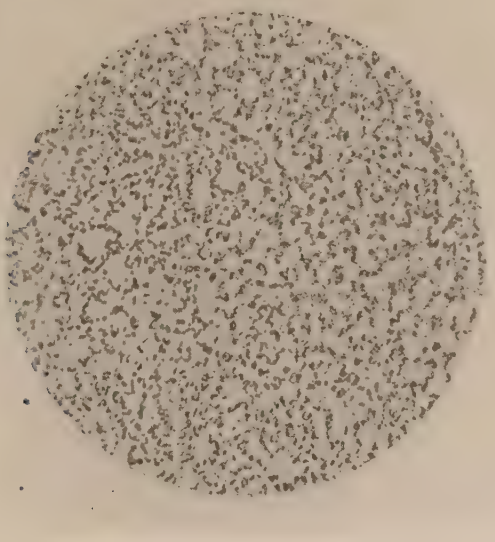


FIG. 34. ALCALIGINES MELITENSIS

The milk secretion may become diminished and is sometimes found to contain flocculi resulting from an inflammatory condition of the udder. On the other hand in many cases there is no detectable physical change in the milk. Arthritis, neuritis, orchitis, keratitis and bronchitis are complicating factors in goats and sheep. Another outstanding feature of the disease in goats and sheep is the high percentage of abortions among affected, pregnant females. Some authors report from 50 to 80 per cent abortions among affected, pregnant females in certain outbreaks. This feature is exceedingly interesting in view of the otherwise close relationship of Alcali-

gines melitensis and Alcaligines abortus. Aside from goats and sheep other lower animals (cattle, horses, swine) are only slightly susceptible.

The natural mode of infection in man is usually through the ingestion of raw goat's milk. It occasionally results, however, through handling affected goats. Further, infection of laboratory workers carrying on bacteriological investigations with Alcaligines melitensis has not been uncommon. In animals the disease is spread through the ingestion of food and water contaminated with the urine or milk of affected animals or carriers.

Malta fever in goats and sheep presents a very important carrier problem. A large percentage of apparently healthy animals in localities where the disease exists are carriers of the infection. In such animals the organism is harbored in the udder and frequently in the blood stream. It is eliminated with the milk and urine.

Morphology and Staining Characteristics. Alcaligines melitensis is a non-motile rod, ordinarily so short and plump as to appear coccoid. Its greatest dimension is usually about 0.4 micron or less although somewhat longer forms are occasionally seen. It does not form spores. It occurs singly and in pairs. Alcaligines melitensis stains well with the common aniline dyes and is Gram-negative.

Cultural Characteristics. Alcaligines melitensis can be isolated direct from the milk and urine and during the febrile stages, at least, from the blood of affected animals. While growth is rather slow the organism can be cultivated on most of the ordinary media. A more luxuriant growth occurs on media containing sheep, goat, horse or cattle serum and nutrose. It is an aerobe and facultative anaerobe growing best at the usual incubator temperature.

Agar. On agar plates small, round, slightly raised, dew-drop-like colonies with a slightly yellowish center, appear after 36 to 48 hours' incubation. The growth of older cultures adapted to artificial cultivation, is more rapid and luxuriant.

Gelatin. The growth of Alcaligines melitensis on gelatin is extremely slow with no liquefaction. Minute, clear colonies develop on gelatin plates after 5 or 6 days' incubation.

Bouillon. Broth is rendered uniformly cloudy after 3 or 4 days with sedimentation occurring after an additional 3 or 4 days.

Litmus Milk. Alcaligines melitensis does not coagulate or acidify milk. Some cultures render the milk slightly alkaline producing a deeper blue.

Potato. Growth on potato is very scanty and of a brownish appearance.

Peptone Solution. Indol is not produced in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites.

Fermentable Media. Alcaligines melitensis does not ferment any of the ordinary fermentable media.

Resistance. Alcaligines melitensis, like Alcaligines abortus, is not a very resistant organism. It is readily destroyed by relatively low temperatures (58° to 60°C). in a few minutes. It will withstand drying for a considerable time, especially if protected from direct sunlight. The ordinary antiseptics in the usual strength solutions are efficacious in rendering it inert.

Serological Reactions. Complement-fixation and agglutination tests are of value in diagnosing cases of Malta fever and in identifying carriers. Because of its relative simplicity the agglutination test is more often employed than the complement-fixation test. Reactions with sera in dilutions of 1:80 or greater are considered diagnostic of the disease. Some specimens will give agglutination in very high dilutions (1:5000 or more). High agglutination titres, however, are more common with sera from human cases than with specimens from the lower animals.

Agglutinin absorption tests are of value in differentiating Alcaligines melitensis from Alcaligines abortus. Then, as pointed out by Buck, Alcaligines melitensis can be isolated in primary culture without particular difficulty which is not the case with Alcaligines abortus (see page 179).

Immunization. Various attempts have been made to immunize against Malta fever through the use of sera and bacterins. The results have not been satisfactory.

ALCALIGINES BRONCHISEPTICUS (BACILLUS BRONCHISEPTICUS, BACTERIUM BRONCHISEPTICUS, BACILLUS BRONCHICANIS, BACTERIUM BRONCHICANIS)

Alcaligines bronchisepticus gained prominence in 1910 when Ferry,[7] and a few months later M'Gowan,[8] reported the organism from cases of distemper and indicated that it was of primary etiological significance. Subsequent work by these investigators strengthened their belief in the organism as the causative factor of canine distemper. Since these reports the significance of Alcaligines bronchisepticus in canine distemper has been the subject of considerable contention. Some investigators agree with Ferry and M'Gowan while others maintain that the organism in question is merely a secondary invader in a disease primarily induced by an unknown, filterable virus. As the matter now stands, it cannot be said that Alcaligines bronchisepticus has been satisfactorily shown to be the primary cause of canine distemper.

[7] Ferry, N. S., Amer. Vet. Rev., July, 1910, p. 499.
[8] M'Gowan, J. P., Jour. Path. and Bact., vol. xv, 1911.

Alcaligines bronchisepticus is found in the bronchial secretion and nasal discharge of animals affected with distemper. In some cases it can be demonstrated in the blood and in fatal cases it can often be isolated from various of the internal organs.

In addition to its presence in canine distemper, Alcaligines bronchisepticus has been isolated from cases of distemper in cats and it has also been found in a respiratory disorder of rabbits known as "snuffles." Further, Alcaligines bronchisepticus has been shown to be responsible for outbreaks of pneumonia in guinea pigs.

Morphology and Staining Characteristics. Alcaligines bronchisepticus is a small rod with rounded ends, the average measuring about 0.50 by 1.5 to

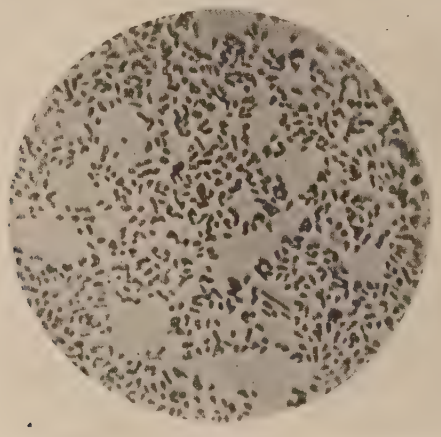


FIG. 35. ALCALIGINES BRONCHISEPTICUS

2 microns. It usually occurs singly but is occasionally seen in pairs. In fluid media there is a tendency toward chain formation. Spores are not formed. The organism is motile by means of peritrichous flagella. It stains with aqueous solutions of the usual aniline dyes but not as readily as most bacteria. Loeffler's methylene-blue gives satisfactory results. The organism is Gram-negative.

Cultural Characteristics. Alcaligines bronchisepticus is an aerobe and facultative anaerobe. It grows best at the usual incubator temperature of 37.5°C. but will develop more slowly at lower temperatures.

Agar. On agar plates small, round, slightly raised, translucent, pin-point colonies appear in 24 hours. These enlarge considerably during the

next 24 hours and gradually become opaque, appearing white with an opalescent sheen. On agar slants a rather thin, moist, glistening, grayish, slightly tenacious growth occurs.

Gelatin. The growth on gelatin is slow with no liquefaction. The colonies on gelatin plates are similar to those on agar. A filiform growth occurs in gelatin stab cultures.

Bouillon. In broth Alcaligines bronchisepticus produces a moderate turbidity in 24 hours. After this a sediment starts to form and increases with the age of the culture, the turbidity likewise increasing. In young cultures the sediment is readily stirred up in a fine cloud by shaking whereas in older cultures the deposit becomes rather viscid and is not broken up so readily. Broth cultures develop a stale or musty odor which increases with the age of the culture.

Litmus Milk. Litmus milk is rendered alkaline by Alcaligines bronchisepticus, the blue becoming more intense, starting in the upper stratum and progressing downward. This change may not start to manifest itself until after 48 or 72 hours, increasing with age.

Potato. The growth of Alcaligines bronchisepticus on potato is usually abundant. After 24 hours a buff or tan colored, moist, sticky, glistening growth appears. As it continues the color becomes deeper with a darkening of the medium. A stale or musty odor is usually marked in potato cultures.

Peptone Solution. Alcaligines bronchisepticus does not produce indol when cultivated in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced.

Fermentable Media. No acid or gas is produced in the fermentable media commonly used.

Resistance. Alcaligines bronchisepticus is not a very resistant organism. In fluid it is destroyed in 20 minutes by a temperature of 55°C. The usual antiseptics are very effective in destroying it. Freezing temperatures have no detrimental effect.

Serological Reactions. Dogs which have distemper will frequently give positive agglutination tests with Alcaligines bronchisepticus antigen. Agglutination values, however, do not run high, ranging usually from 1:60 to 1:200, rarely going to 1:1000.

Biological Therapy and Prophylaxis. Various bacterins, sera, etc., prepared from Alcaligines bronchisepticus, have been produced and are at present used by a considerable number of veterinarians for the treatment and prevention of canine distemper. It cannot be said that the results from such products are very satisfactory.

CHAPTER XIX

Order—Eubacteriales, Family—Bacteriaceae (Continued), Tribe—Pasteurelleae. Organisms of the Genus Pasteurella

Under the tribe "Pasteurelleae" the Committee of the Society of American Bacteriologists have grouped in one genus (Pasteurella) the bipolar-staining organisms of the well-known *hemorrhagic septicemia group* (Hueppe[1]).

The genus "Pasteurella" was first suggested by Trevisan in 1887 and while accepted by some bacteriologists it did not come into general use. The Committee of the Society of American Bacteriologists, in recognizing it in their system of classification, give the following specifications for the genus:

> Aerobic, facultative. Powers of carbohydrate fermentation slight; gas produced. Gelatin not liquefied. Parasitic, frequently pathogenic, producing plague in man and hemorrhagic septicemia in the lower animals.

From the standpoint of veterinary medicine this is one of the most important groups of organisms in bacteriology.

Organisms of the Pasteurella genus are widely distributed in nature. They are very frequently found in the upper air passages of normal cattle, horses, swine, sheep, dogs and cats. Less frequently they are found as normal inhabitants of the intestinal tract. Thus, in a way, they are more or less facultative-pathogenic bacteria, being present in normal animals and only producing disease when the normal resistance of the animal is lowered, or following a particular increase in the virulence of the organism which, in some unknown way, seems to occur. While originally more or less of a saprophyte, after attaining initial virulence further passage of some strains frequently increases the virulence to a very marked degree. On the other hand, there is evidence which tends to indicate that, in some instances, after a single disease-producing passage through a host a strain may again assume its saprophytic nature.

Among the various members of the Pasteurella group are those responsible for hemorrhagic septicemia in cattle, sheep, horses and buffalo. Other members are etiological factors in swine plague, fowl cholera and tularemia.

[1]Hueppe, Berl. Klin. Wochen., 1886.

Further, members of this group are known to be important secondary agents in certain diseases of horses, cattle and swine.

In the past it has been customary to refer to an organism of the hemorrhagic septicemia group as "Bacillus bipolaris septicus," when it was not desired to indicate a particular species and when designating a species the term "Bacillus bovisepticus" or "Bacillus equisepticus," etc., was employed, according to the species of animal from which the organism was isolated. Thus, the group includes the hemorrhagic septicemia bacteria formerly known as "Bacillus bovisepticus," "Bacillus equisepticus," "Bacillus suisepticus," "Bacillus ovisepticus," "Bacillus bubalsepticus" and "Bacillus avisepticus." The "Bacterium tularense" of McCoy and Chapin, the causative agent of tularemia, a plague-like disease of squirrels and rabbits is also included in this genus.

While it has been customary to designate a separate species of Pasteurella for practically every species of animal from which the organism has been isolated, there is every indication that in some cases the particular organism is transmitted from one species to another.

Hemorrhagic septicemia varies somewhat in the different animals but in the main it manifests itself as an acute septicemia giving rise to high temperature, impaired heart action, marked respiratory disturbance, prostration and frequently diarrhea which may be of a bloody character. Hemorrhages occur in various parts of the body. Petechial hemorrhages of the serous and mucous membranes and various organs are particularly common. The lymphatics are usually markedly swollen and congested. Other changes frequently noted are edema of the subcutaneous tissue, blood extravasations into the subcutaneous tissues, inflammation of various joints, serofibrinous inflammation of the serous membranes, necrotic pneumonia, etc.

The disease is particularly common in cattle, occurs frequently in sheep, and in hogs is seen in the form of "swine plague." Bison are also very susceptible. The equine species is not often affected, but when hemorrhagic septicemia does occur in horses and mules it usually manifests itself in a very acute and severe form.

While slight cultural variations have been described for different members of the hemorrhagic septicemia group, it is practically impossible to differentiate different species by the ordinary bacteriological procedures. Pasteurella tularensis and Pasteurella pestis possess characteristics which make their identification readily possible.

PASTEURELLA BOVISEPTICA (BACILLUS BOVISEPTICUS)

Pasteurella boviseptica is the causative agent of hemorrhagic septicemia of cattle, a disease of tremendous importance because of its relative frequency of occurrence and its high mortality.

In 1878 Bollinger[2] reported the observation of a fatal disease of wild deer and hogs near Munich. Subsequently this new malady, which later proved to be hemorrhagic septicemia, made its appearance in cattle and to a lesser degree in domestic hogs and horses. While Bollinger recognized the infectious character of the disease, it remained for Kitt,[3] in 1885, to discover the etiological factor. Hemorrhagic septicemia of cattle has subsequently been recognized in various parts of the world including the United States where it is quite common and, hence, of considerable economic importance.

The general changes produced by Pasteurella boviseptica are petechial hemorrhages and ecchymoses in the serous and mucous membranes and various organs. An edematous or exanthematous form of the disease is recognized in which a hemorrhagic, gelatinous infiltration of the subcutaneous tissues, particularly those of the head and neck region, occurs. The tongue in such cases is usually edematously swollen and reveals hemorrhages when sectioned. The regional glands are acutely swollen and hemorrhagic. In some cases the lungs are simply congested and show small, hemorrhagic areas of varying size and number. However, in the more pronounced, pectoral form of the disease a marked involvement of the lungs and pleurae is noted. In such instances the lungs are markedly hyperemic, edematous and may contain hepatized and necrotic areas. The pleura is greatly inflamed, hemorrhagic, thickened and covered with fibrinous deposits.

In some respects hemorrhagic septicemia is somewhat like anthrax and, hence, might be confused with it. In this connection it is noteworthy that the spleen is not greatly enlarged, softened and dark in hemorrhagic septicemia which, in a measure, serves to differentiate it from anthrax.

The disease runs a course varying from a few hours to 6 or 7 days. The mortality is exceedingly high it being estimated that 90 per cent or more of affected animals succumb.

Pasteurella boviseptica can be isolated from the blood during the height of the disease, and after death, from the blood, various organs and affected tissues.

Morphology and Staining Characteristics. Pasteurella boviseptica is a

[2] Bollinger, Ub. eine neue Wild- und Rinderseuche usw. München, 1878.

[3] Kitt, Sitzgsb. d. Ges. f. Morph., München, 1885.

short, non-motile, non-sporulating, ovoid rod with rounded ends, the average measuring about 0.50 by 0.80 to 1 micron. It usually occurs singly, occasionally in pairs and rarely in short chains.

The organism stains with the usual aniline dyes and is Gram-negative. The manner in which it stains is characteristic of the group. In suitable preparations it takes the dye only at the poles (bipolar), the middle of the organism remaining unstained. This bipolar staining is more pronounced with organisms in the blood, exudates and tissues than with cultures. As a rule, the bipolar-staining property of the organism can seldom be demonstrated satisfactorily with preparations made direct from cultures. The characteristic staining is best demonstrated by using a diluted dye (carbol-

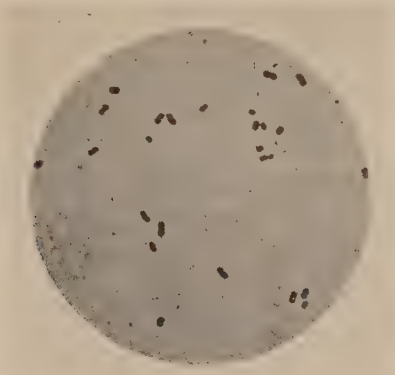


Fig. 36. Pasteurella boviseptica (Peritoneal Fluid)

fuchsin 1:10) and not staining too long. Organisms in blood film stained by Wright's method, show the bipolar-staining property very satisfactorily.

Cultural Characteristics. Pasteurella boviseptica is an aerobe and facultative anaerobe. It grows best at the usual incubator temperature of 37.5°C. but will propagate more slowly at lower temperatures. Media of a slightly alkaline reaction are best suited for its cultivation. The organism will grow upon practically all of the ordinary types of culture media but not luxuriantly. It grows particularly well on medium containing blood serum or whole blood.

Agar. On agar plates, small, transparent, slightly raised colonies develop after 24 hours. These rapidly become glistening white and on examination with a hand lens will be found to be granular. On agar slants a grayish-white, slightly raised, glistening growth develops. The growth of Pasteur-

ella boviseptica, as well as other members of the Pasteurella genus, tends to be of a pronounced sticky or viscid character.

Gelatin. The colonies on gelatin plates are similar to those on agar Gelatin stab cultures show a white growth along the line of the stab. There is no liquefaction of the medium.

Bouillon. When cultured in broth Pasteurella boviseptica causes a uniform turbidity with the subsequent formation of a sediment which is usually of a very viscid character.

Litmus Milk. Litmus milk is rendered acid by Pasteurella boviseptica. No coagulation occurs.

Potato. Pasteurella boviseptica will not grow on potato unless the same is rendered neutral or slightly alkaline. The growth on such medium is scanty, slightly raised and of a grayish-white color.

Peptone Solution. A small amount of indol is produced in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites.

Fermentable Media. Some strains of Paseurella boviseptica produce a small amount of acid from saccharose, dextrose, galactose and mannite. The results obtained with fermentable media are variable.

Pathogenicity. Pasteurella boviseptica is pathogenic for mice, rabbits and guinea pigs. The mouse and the rabbit are more susceptible than the guinea pig. The inoculation is best given intraperitoneally to mice and intravenously to rabbits. Where guinea pigs are employed the injection should be made intraperitoneally.

Blood, exudates or tissue emulsion from affected animals are more virulent than cultures. Adherents of the aggressin theory ascribe this particular virulence of such material to the presence of aggressins.

Cultures of Pasteurella boviseptica, even though recently isolated, rarely produce death when inoculated into normal cattle or other large animals.

Resistance. Pasteurella boviseptica is not a very resistant organism. A temperature of 58°C. will destroy it within 20 minutes. Of the ordinary antiseptics bichloride of mercury is particularly effective, a 1:5000 solution destroying the organism in one minute. Phenol in 5 per cent solution, liquor cresolis compound in 3.5 per cent solution, formalin, etc., are effective in short periods of time.

Serological Reactions. While work with complement-fixation and agglutination tests has been done in hemorrhagic septicemia the tests have not come into practical use.

Immunization. Considerable work has been done with various types of

biological products in hemorrhagic septicemia of cattle. Bacterins containing killed organisms, vaccines containing living or attenuated bacteria, aggressins, antisera, etc., have been employed in the control of the malady. There is considerable disagreement regarding the results obtained with these various agents. The author's observations indicate that suspensions of killed bacteria (bacterins) are the least valuable for immunization against hemorrhagic septicemia. Suspensions of living bacteria (vaccines) which have become attenuated in virulence have given fairly satisfactory results Recently considerable interest has been manifested in aggressin immunization against hemorrhagic septicemia. It will be recalled that some of the early work of Bail, Weil, etc., on aggressins was done with a member of the Pasteurella group (Pasteurella avicida).

Gochenour,[4] in 1924, isolated an exceedingly virulent strain of the hemorrhagic septicemia organism from American buffalo (bison) and subsequently produced an aggressin from calves succumbing to inoculations of the culture. This product has been used rather extensively from the time of his report up to the present and indications are that it is giving excellent results.

The results from immune serum in the treatment of hemorrhagic septicemia have been variable. In the main they have not been satisfactory.

PASTEURELLA EQUISEPTICA (BACILLUS EQUISEPTICUS)

Pasteurella equiseptica is not often found as a primary inciting agent of disease in the equine species. Rarely, however, it does produce pure hemorrhagic septicemia in horses and mules. As already mentioned, when hemorrhagic septicemia does occur in the equine species it manifests itself in a very acute form.

Pasteurella equiseptica is principally of importance as a secondary invader in equine contagious pleuropneumonia. Several investigators, notably Babes and Lignières, considered Pasteurella equiseptica as the primary etiological agent of contagious pleuropneumonia rather than a secondary factor. The bulk of evidence, however, indicates that the organism is not the primary cause of the disease but an important secondary agent.

Exceptionally Pasteurella equiseptica can be demonstrated in the blood and nasal discharge of horses suffering from contagious pleuropneumonia. On the other hand, at autopsy the organism can usually be demonstrated in large numbers in the lung tissue in the red hepatized portions and in the hemorrhagic areas. In other portions of the lungs and in the pleural fluid Pasteurella equiseptica is found only in small numbers or may be entirely

[4] Gochenour, Jour. A. V. M. A., N.S., vol. xviii, no. 4, July, 1924.

absent. The organism is ordinarily found in the affected lung tissue in association with other secondary invaders, especially Streptococcus equi.

Morphology and Staining Characteristics. In its morphology, staining and cultural characteristics Pasteurella equiseptica is practically identical with Pasteurella boviseptica. The organism may possibly appear a little smaller than the causative agent of bovine hemorrhagic septicemia and in cultures there seems to be a tendency to occur in diplococcic form.

Resistance. Pasteurella equiseptica is similar in every way to Pasteurella boviseptica in its attitude towards heat and chemical disinfectants.

Immunization. Various biological products have been prepared and utilized in the treatment and prevention of contagious pleuropneumonia. In the treatment of the disease serum from animals which have received numerous injections of considerable amounts of cultures of Streptococcus equi and Pasteurella equiseptica is employed. For prophylactic purposes, serum, bacterins and vaccines have been used. During the world war these various agents were tried out in the United States Army on rather a large scale. The results were very disappointing.

PASTEURELLA SUISEPTICA (BACILLUS SUISEPTICUS)

For a number of years hemorrhagic septicemia of hogs, or swine plague, due to Pasteurella suiseptica, was confused with hog cholera, a disease caused by a filterable virus. As Pasteurella suiseptica has been definitely demonstrated to play a secondary rôle in hog cholera, it can be readily understood how such confusion existed.

In 1886 Loeffler[5] demonstrated the organism we now recognize as Pasteurella suiseptica in the septicemic form of swine plague. Shortly following this observation Schultz demonstrated the same organism in the pectoral form of the disease. Confirmation of the work of Loeffler and Schultz was soon forthcoming in both Europe and America and it is now definitely established that swine plague occurs as a distinct disease apart from hog cholera.

Swine plague manifests itself either as a peracute, hemorrhagic septicemia in which extensive pulmonary changes have not had time to develop, or as a somewhat less acute affection in which marked pulmonary involvement with hepatization and necrotic areas are present. Further, a chronic form of the disease is recognized in which the animal becomes greatly emaciated. In such cases autopsy reveals old necrotic foci in the lungs, caseous foci in various lymph glands, joint involvement, etc.

[5] Loeffler, Arbeiten aus dem Kaiserlich. Gesundheitsamte, i, 51, 1886.

Pasteurella suiseptica can be readily isolated from the blood, and from lesions in the lungs, kidneys and other organs and lymph glands of animals which have succumbed to the affection.

Morphology and Staining Characteristics. The morphology and staining characteristics of Pasteurella suiseptica are essentially the same as those described for Pasteurella boviseptica. The organism has, however, a slight tendency to appear a little larger than the bovine variety and in preparations from old lesions they frequently appear as swollen, vacuolated bodies with only a peripheral ring taking the stain.

Cultural Characteristics. In practically all respects the cultural characteristics described for Pasteurella boviseptica are applicable to Pasteurella

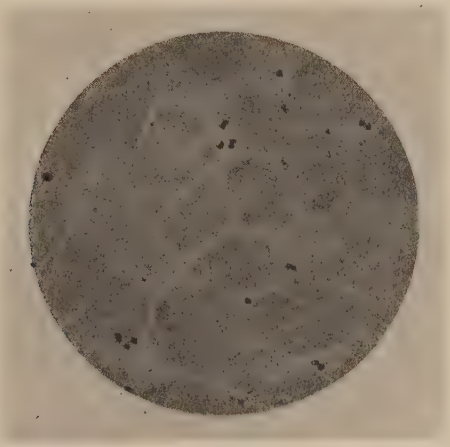


Fig. 37. Pasteurella suiseptica (Blood)

suiseptica. The latter organism probably grows a little better under anaerobic conditions than some other members of the genus. Further, Pasteurella suiseptica, as a rule, will not grow on potato. Litmus milk is rendered acid without coagulation.

Resistance. In its tenacity Pasteurella suiseptica is similar to the other members of the genus, the information given for Pasteurella boviseptica being applicable to this organism.

Biological Therapy and Prophylaxis. Killed and attenuated cultures have been employed for the immunization of hogs against swine plague with rather discouraging results. The experimental work of various investigators tends to indicate that where any appreciable immunity is obtained

with a product made from one strain of Pasteurella suiseptica the animal is still susceptible to different strains. Thus, where bacterial products are employed a number of strains should be used in their preparation. The same applies to the preparation of anti-serum.

PASTEURELLA AVICIDA (BACILLUS AVISEPTICUS)

Pasteurella avicida is the etiological agent in fowl cholera, one of the most important of all diseases of fowl.

While the contagious nature of fowl cholera was recognized as early as 1851, the causative organism was not discovered until 1878. In that year Perroncito[6] saw the organism in the blood of affected birds but mistook it for a diplococcus. During the next two years (1879 and 1880) both Toussaint[7] and Pasteur[8] confirmed Perroncito's findings and succeeded in cultivating the organism. Pasteur's work with the fowl cholera organism was epoch-making. His fundamental experiments in immunization with attenuated cultures were carried out with the organism of fowl cholera.

Fowl cholera is a markedly acute disease with a high mortality. It is characterized by depression, extreme weakness, difficult respiration and profuse diarrhea. Autopsy reveals an acute, hemorrhagic enteritis, marked pulmonary congestion with hemorrhagic areas in the lung tissue and hemorrhages in the serous membranes, including the epicardium.

Chickens, ducks, geese and pigeons are all susceptible to the disease. Experimentally, in addition to birds, mice and rabbits are susceptible. Guinea pigs are less susceptible but intraperitoneal injections are usually fatal.

Pasteurella avicida can be readily isolated from the blood, various organs and intestinal contents of affected birds.

Morphology, Staining and Cultural Characteristics. In its morphological, staining and cultural characteristics Pasteurella avicida corresponds in general to the other members of the genus. Thus, the characteristics described for Pasteurella boviseptica apply, in the main, to this organism. Pasteurella avicida, however, appears to be a little smaller than other members of the group. In saccharose broth the majority of strains produce acid. Litmus milk is not changed and no growth ordinarily occurs on potato medium.

Resistance. In tenacity Pasteurella avicida corresponds to Pasteurella boviseptica and other members of the genus.

[6] Perroncito, Arch. f. Wessenschaftl und fraktische Thierheilkunde, 1879, v, 22.
[7] Toussaint, Recueil de médecine veterinaire (Alfort), 1879, 946.
[8] Pasteur, Comptes rend. de l'acad. des sci., 1880.

Immunization. Vaccines and bacterins have been employed in attempts to immunize fowl against cholera. While some reports indicate fair results others are discouraging.

PASTEURELLA BUBALSEPTICA (BACILLUS BUBALSEPTICUS)

Hemorrhagic septicemia of buffalo (water buffalo) was reported from Italy in 1886 by Oreste and Armanni.[9] The disease was subsequently recognized in Russia, Hungary, East Indies, Indo-China, Egypt, etc. Pasteurella bubalseptica, the causative agent, corresponds to Pasteurella boviseptica in its morphological, staining and cultural characteristics.

PASTEURELLA PESTIS (BACILLUS PESTIS)

One of the most dreaded of all scourges of man is bubonic plague, caused by Pasteurella pestis. In Europe, during the fourteenth century, millions of persons succumbed to the so-called "black death." The disease has also occurred in various parts of Asia, Australia, Africa and America. Fortunately, as a result of our improved knowledge of infectious diseases, particularly as regards their cause and transmission, plague is not the terror that it was during the middle ages but it still frequently takes a tremendous toll in lives in certain countries, particularly India.

Pasteurella pestis was identified as the cause of plague in 1894 by Kitasato[10] and Yersin[11] working independently. The reports of both of these investigators were published in July, 1894, Kitasato's paper preceding Yersin's by about three weeks.

While plague is not a disease of the domesticated animals, it is of interest to the veterinary profession because it is primarily a disease of rodents. While the rat is the chief offender, other rodents, particularly squirrels, are serious factors in the perpetuation of the disease and its subsequent transmission to the human family. In the United States the California ground-squirrel has been an important and difficult problem in plague control. The marmot, the Siberian tarbagan and tree-squirrels have likewise proved important factors in plague outbreaks.

Experimentally white rats, guinea pigs, mice, rabbits and monkeys can be infected. Guinea pigs and white rats are particularly suitable for experimental infection. The disease can be produced by feeding the organism as well as by inoculation. Infection frequently results where the organism is

[9] Oreste and Armanni, Rev. Vet. Jhb., ii, 1887.
[10] Kitasato, The Lancet, 1894.
[11] Yersin, Ann. de l'Inst. Pasteur, viii, 1894.

merely rubbed on the unbroken skin or mucous membrane. If a slight scratch or abrasion is made, infection is sure to result.

The disease in rodents is characterized by enlargement of the glands (buboes), especially those of the neck region, a peculiar dark purplish-red appearance of the abdominal muscles, pinkish appearance of the subcutaneous tissue, and the presence of hemorrhagic-edematous areas, particularly in the submaxillary region. The pleural cavity usually contains a considerable quantity of clear fluid. The liver usually has a so-called "granular" appearance due to the presence of minute, grayish or yellowish-white granules. The spleen is usually enlarged.

While plague in rats is ordinarily an acute affection chronic cases occasionally occur in which rats harbor the infection in old lesions in the spleen, liver and various glands. Such rats are a menace even though the organism is not present in the circulation or is not eliminated from the body. If such rodents die of some other cause, while thus harboring Pasteurella pestis, their carcasses may be eaten by susceptible rats and acute cases of the disease started.

McCoy[12] found ground squirrels harboring Pasteurella pestis in purulent foci in one or more lymph glands with no additional evidence of disease.

While plague can be transmitted from animal to animal through ingestion of infected food or water, the common mode of transmission is by means of an intermediate host, the flea. The latter becomes infected when it draws blood from an infected rodent. After its host dies the flea soon leaves it and seeks another. The bacteria ingested multiply enormously in the flea's digestive tract and are deposited on the skin of the new host (animal or man) in feces or regurgitated material deposited by the flea near the point of bite during the period of feeding. Infection can occur through the wound made by the bite or through subsequent scratching.

In man pneumonic plague, a highly fatal form of the disease, is commonly communicated direct from man to man as a result of organisms sprayed in the air during coughing.

The flea, commonly serving as the intermediate factor in the spread of plague in Europe and the United States, is Ceratophyllus faciatus. In India the flea Xenopsylla cheopis is the common offender.

Morphology and Staining Characteristics. In its morphology and staining characteristics Pasteurella pestis resembles the other members of the Pasteurella group. In preparations from buboes, blood, spleen, liver, etc., the typical bipolar-staining organism will be seen in large numbers with occasional coccoid, large oval, and club-shaped forms. Preparations made from

[12] McCoy, G. W., U. S. Pub. Health Bul. 43, U. S. P. H. and M. H. Service, April, 1911.

cultures grown on a 3 per cent salt agar show marked pleomorphism. Large clubs, dumb-bell, coccoid and other involution forms are noted when cultivated on such medium. The organism usually occurs singly or in pairs but tends to form short chains in fluid media.

Cultural Characteristics. The plague organism is not unlike the other members of the genus in its main cultural characteristics. Pasteurella pestis, however, is more of a strict aerobe than some members of the genus. As already indicated, marked pleomorphism is noted when the organism is grown on salt agar. In bouillon a characteristic "stalactite" growth occurs if the cultures are allowed to develop without much handling. A pellicle forms on the surface of such medium and thread-like growths hang down

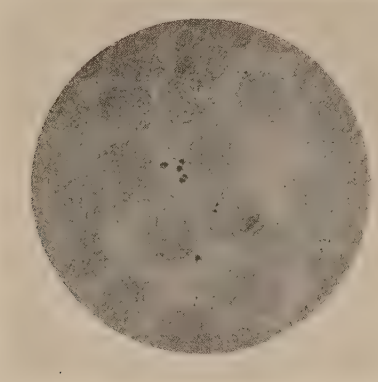


FIG. 38. PASTEURELLA PESTIS (RAT BLOOD)

from same in an otherwise clear medium. A granular sediment forms at the bottom of the tube and the same type of deposit frequently occurs on the sides of the tube. Litmus milk is slightly acidified. On potato a scanty, grayish growth occurs.

Resistance. Same as Pasteurella boviseptica. The organism is very susceptible to drying.

PASTEURELLA TULARENSIS (BACTERIUM TULARENSE, BACILLUS TULARENSE)

McCoy,[13] in examining ground squirrels from Tulare County, California, for evidence of bubonic plague, found in some of them lesions which might readily be taken for those of plague but which were, in reality, due to another

[13] McCoy, U. S. P. Health Bul. No. 43, April, 1911.

organism. The organism too, in many ways, was like Pasteurella pestis but was sharply differentiated from the latter by its failure to grow on the usual culture media employed for the cultivation of the plague bacterium. In fact, for some time it was impossible to cultivate the organism but McCoy and Chapin[14] finally succeeded in growing it upon a medium made of egg yolk. They named the organism "Bacterium tularense," adopting such name because it was in squirrels from Tulare County, California, that the disease was first noted. Under the present classification it is known as "Pasteurella tularensis."

Subsequently, Francis of the Hygienic Laboratory, United States Public Health Service, showed that Pasteurella tularensis is responsible for a disease of considerable significance in man. He has designated the malady "Tularemia." In addition to squirrels he found that the jack-rabbits in certain localities west of the Mississippi River, and the cotton-tail rabbits in some sections east of the Mississippi, are frequently found infected with Pasteurella tularensis. He further found that the so-called "deer-fly fever" in man is tularemia, resulting from the bite of the fly Chrysops discalis which has previously bitten an infected rabbit.

Experimental evidence indicates that other biting insects such as ticks, lice, fleas, bed-bugs, etc., can transmit the disease. Further, market people dealing in rabbits have frequently become infected through the handling of carcasses of such animals. The disease is commonly known to market dealers as "rabbit fever." Laboratory infections among investigators working with the organism have been common.

Tularemia among rabbits was first demonstrated in animals from Utah and adjoining states. Present evidence, however, indicates that it is by no means limited to that locality. Francis states that the disease has been authentically reported from California, Utah, Wyoming, Idaho, Oregon, Colorado, Ohio, Indiana, Tennessee, North Carolina, Montana, New Mexico, Virginia, West Virginia and the District of Columbia.

Pasteurella tularensis is characterized by a marked ability to infect. Apparently the organism is capable of penetrating the unbroken skin, certainly it will infect when applied to a slightly irritated area of the skin.

Of the experimental animals guinea pigs, rabbits, mice and monkeys are susceptible. The disease in animals is characterized chiefly by lesions in the liver, spleen, and lymph glands. The liver and spleen are usually "sprinkled" with small, white, necrotic foci which stand out particularly well when examined in the sunlight. The organism can be isolated from the liver, spleen, affected lymph glands, and the blood.

[14] McCoy and Chapin, Jour. Inf. Dis., x, 1912.

Morphology and Staining Characteristics. Pasteurella tularensis is a small, Gram-negative, non-motile rod measuring about 0.20 micron in thickness and from 0.3 to 0.70 micron in length. Both bacillary and coccoid forms occur. It may occur coccoid in one culture and in a single transfer assume

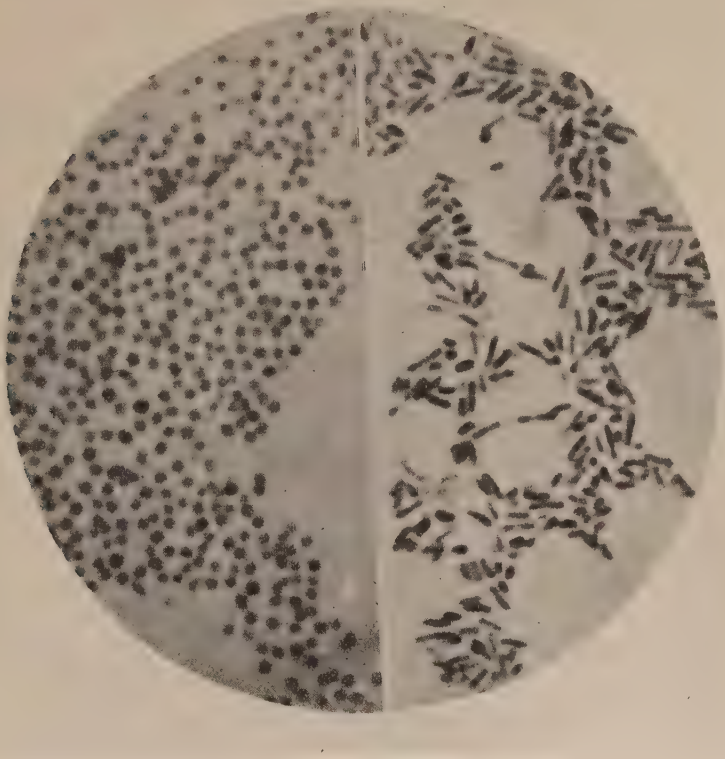


FIG. 39. PASTEURELLA TULARENSIS

Note change from coccus-form to rod-form in single transfer on artificial medium (Photomicrograph by Major George R. Callender, Army Medical Museum. Courtesy of Surgeon E. Francis, U. S. P. H. S.)

a rod-shaped form (Fig. 39). The organism usually occurs singly. Of the aniline dyes carbol-fuchsin and gentian-violet are best suited for staining the organism.

Cultural Characteristics. Pasteurella tularensis is an aerobe and grows best at the usual incubation temperature of 37.5°C. but will propagate at

slightly lower temperatures. As indicated above, it is very fastidious in its media requirements. It can be isolated on a medium consisting of coagulated egg yolk or better on Francis' serum-glucose-cystine-agar (see page 64 for preparation of medium). On such medium minute, grayish-white, viscous colonies develop. Best results are obtained where a piece of tissue (spleen) containing the organism is rubbed over the surface of the medium.

Pathogenicity. The disease can be readily transmitted to experimental animals by shaving the abdomen rather roughly so as to cause a slight amount of irritation and then rubbing a piece of spleen or liver tissue from a case of the disease over the shaved area of the guinea pig or rabbit. Culture can be inoculated in this manner or be injected.

Serological Reactions. Agglutinins and complement-fixing bodies occur in the disease. The agglutinating test is of particular value for the diagnosis of the disease in man.

Resistance. The organism is similar to others of the genus in this respect.

CHAPTER XX

Order—Eubacteriales (Continued), Family—Bacilliaceae. Organisms of the Genus Bacillus

The family Bacillaceae is composed of two genera—Bacillus and Clostridium. The specifications for the genus Bacillus, as given by the Committee of the Society of American Bacteriologists, are:

> Aerobic forms. Mostly saprophytes. Generally liquefy gelatin. Often occur in long chains and form rhizoid colonies. Form of rod usually not greatly changed at sporulation. Young organisms are Gram-positive.

As indicated, most of the organisms falling in this genus are non-pathogenic. Of the few species that are pathogenic there is one of particular importance in veterinary medicine. That is Bacillus anthracis, the etiological factor in anthrax. Then, Bacillus subtilis, while non-pathogenic is, as indicated below, worthy of mention.

Bacillus subtilis (hay bacillus)

While not pathogenic and of no special importance in veterinary medicine, Bacillus subtilis is briefly referred to here because it is the type species of the genus Bacillus and because it is one of the organisms universally used in instruction in bacteriology. Further, in some respects the hay bacillus is similar to Bacillus anthracis and has been mistaken for such organism by casual observers. Bacillus subtilis is widely distributed in nature being a normal inhabitant of soil, hay, straw, dust, etc.

Morphology and Staining Characteristics. Bacillus subtilis is a large, motile, sporulating rod. In size it averages about 0.8 to 1.2 microns in thickness and from 1.5 to 3.5 microns in length. It occurs singly and in short and long chains. The spore is usually centrally located but may be eccentric. Its motility is due to peritrichous flagella. It stains well with the ordinary aniline dyes and is Gram-positive.

Cultural Characteristics. The organism is aerobic, consuming large quantities of oxygen. This oxygen-consuming property of the hay bacillus is sometimes made use of in cultivating organisms which require a lowered oxygen tension. Cultures of Alcaligines abortus, for example, can be cultivated in original culture when grown in symbiosis with cultures of Bacillus

subtilis. It grows well at the usual incubator temperature but will propagate between wide temperature limits (12° to 52°C.) It can be readily cultivated on practically all of the common media.

Agar. On agar plates grayish-white, granular colonies with serrated margins appear. On agar slants a luxuriant, dry, wrinkled growth develops.

Gelatin. Colonies on gelatin appear white, later becoming darker and granular with liquefaction of the surrounding medium. In gelatin stab cultures a grayish-white growth develops on the surface and liquefaction proceeds from the surface downward.

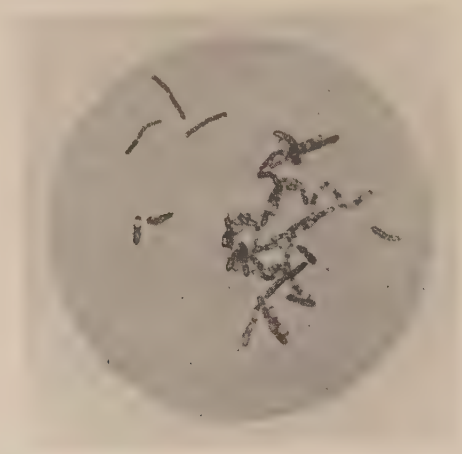


FIG. 40. BACILLUS SUBTILIS

Bouillon. Bouillon is rendered uniformly turbid with pellicle formation and considerable sedimentation.

Litmus Milk. Bacillus subtilis renders litmus milk alkaline and peptonizes it.

Potato. The growth of the hay bacillus on potato is very luxuriant. It occurs as a grayish, nodular growth with a tendency to subsequently take on a pinkish tinge.

Peptone Solution. Indol is not produced in Dunham's peptone soution.

Nitrate Solution. Nitrates are not reduced to nitrites.

Fermentable Media. Bacillus subtilis produces acid from dextrose and sucrose.

Resistance. Bacillus subtilis in the vegetative form is destroyed within 20 minutes by a temperature of 60°C. The spores, however, are extremely resistant. withstanding boiling for a number of minutes.

BACILLUS ANTHRACIS

Anthrax, charbon or splenic fever, caused by Bacillus anthracis, is an important and one of the oldest of the infectious diseases met with in veterinary medicine. Further, anthrax is of considerable historical importance because it figured conspicuously as a subject for study by the early investigators who laid the foundation of modern bacteriology. Anthrax was the first of all infectious diseases of man and the lower animals shown to be caused by a specific microörganism.

It is interesting to note that the etiology of anthrax was established by steps in which several investigators played important parts. The infectious nature of anthrax was demonstrated as early as 1836 by transmission experiments conducted by Erlert. However, at that time nothing was known of the nature of the causative factor. In 1855 Pollender[1] reported the observation of the rod we now know as the anthrax bacillus, in the blood of animals dead of anthrax but he did not prove its etiological significance. In 1857 Brauell,[2] who had been working independently, likewise reported the observation of the anthrax bacillus. Delafond, in 1860, came out with the conclusion that the rods found in the blood of animals dead of anthrax were bacteria. In 1863 Davaine[3] announced that he could regularly produce anthrax by inoculating susceptible animals with blood containing the bacilli, even when such blood was greatly diluted, and that the disease could not be produced with blood which did not contain the rods. This, obviously, was a tremendous argument in favor of the bacillus as the causative factor. Finally in 1876 Koch[4] established, beyond all doubt, the etiological significance of the organism by isolating it in pure culture and producing the disease through the inoculation of such culture.

Anthrax is principally a disease of herbivorous animals, although hogs, dogs, cats and various wild animals occasionally become affected. Man is also susceptible, the disease in the human family being commonly designated as "malignant pustule" and "woolsorter's disease." Birds and fowl are practically immune.

Of the domestic animals sheep are most susceptible. Cattle come next when natural infection is considered but they are not very susceptible to artificial infection by the subcutaneous injection of virulent culture. Fre-

[1] Pollender, Vierteljschr. f. ger. Med., 1855.

[2] Brauell, Virchow's Archiv. für path. Anatomie u. Physiol. und für Klin. Medizin, 1857, xi.

[3] Davaine, Comptes rend. de l'acad. des. sci., 1863.

[4] Koch, Beitr. z. Biol. d. Pflanzen, Cohn, 1876.

quently only a local reaction follows such injection. However, intravenous injections and the feeding of cultures readily produce the disease. Next in order of susceptibility are the horse and mule. Hogs are rather resistant but occasionally become affected, the disease in such animals usually developing as a localized affection of the throat region, giving the animal the appearance of having mumps. Of the experimental animals mice are most susceptible. Rabbits and guinea pigs are also susceptible while white rats are highly resistant.

Blood, body fluids or tissue extracts from cases of anthrax are particularly virulent when inoculated into susceptible animals. Such material is more virulent than cultures.

Anthrax in herbivora occurs as an acute, febrile affection characterized by depression, weakness, difficult respiration, cyanotic mucous membranes which are frequently studded with petechiae, bloody diarrhea, and sometimes edematous swellings in certain regions of the body, particularly in the neck, sternum, flank and lumbar regions. The mortality is very high, most affected animals succumbing to the disease. On postmortem examination the chief lesions noted are gelatinous infiltrations and extravasations of blood in the subcutaneous tissues, hemorrhages in various parts of the body, particularly in the serous membranes and an acute swelling of the spleen. The condition of the spleen is usually characteristic of the disease. It is much enlarged, hemorrhagic, and the pulp darkened and softened to such an extent as to be semifluid. The liver and kidneys are congested, swollen and reveal evidence of parenchymatous degeneration. The intestinal tract usually reveals a severe enteritis with hemorrhagic areas of varying size. The lungs are congested and, as a rule, contain small hemorrhages. Lymph glands appear swollen and on section are found to be congested and hemorrhagic. The blood is a very dark red and fails to coagulate.

The anthrax organism can be readily isolated from the various organs, glands, edematous fluids and blood of animals which have succumbed to the disease. In the living animal, however, the organism cannot be demonstrated in the circulating blood until just a few hours before death.

The anthrax bacillus varies in virulence according to the character of its mucin-like capsule. The more dense and less soluble the capsule the greater the virulence of the organism. When an animal becomes infected the organisms entering the body localize at or near the point of entry when introduced through the skin, or, if infection takes place through the alimentary canal, the spores germinate in the intestines and the resulting bacilli pass into the lymph spaces where they localize for propagation. At the points of localiza-

tion the anthrax organism attempts its propagation, hindered, of course, by the defensive elements of the body. Avirulent or only slightly virulent organisms succumb to the defense forces of the animal. In the case of virulent, heavy-capsuled bacilli, capsular substance is liberated as a result of the destruction of some of the bacteria by anthracocide substances and

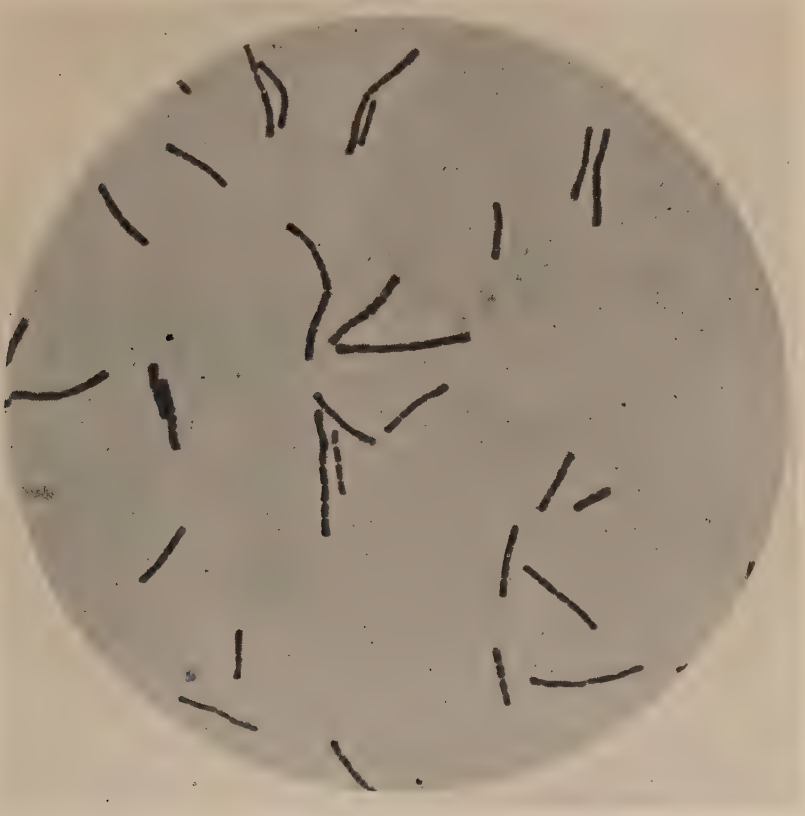


FIG. 41. BACILLUS ANTHRACIS (IN TISSUE EXUDATE)

phagocytes. This liberated material tends to neutralize the anthracocide substances and gives rise to a negative chemotaxis. Under such conditions the surviving anthrax bacilli are able to multiply with less interference, producing progeny with thick capsules. Following this rapid multiplication the bacilli have little difficulty in migrating from the point of localization to various parts of the body, producing the specific disease.

Morphology and Staining Characteristics. Bacillus anthracis is a large, non-motile, sporulating rod averaging about 1.50 microns in thickness and varying from 4 to 10 microns long. The organism forms long chains, particularly in artificial culture. In the blood and body fluids of affected animals somewhat shorter chains, single and paired forms are observed. The ends of the bacillus are either square-cut or slightly concave. Rounded ends are not characteristic of the anthrax organism. The anthrax bacillus is well known for its mucin-like capsule which is well developed in virulent strains, particularly when present in the blood or body fluids. In culture

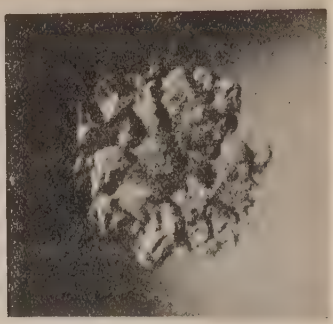


Fig. 42 Fig. 43

Fig. 42. Bacillus anthracis Showing Capsules

Fig. 43. Colony of Bacillus anthracis on Agar

the capsule is best demonstrated when the organism is grown in milk or in media containing serum or other albuminous material.

The spores of Bacillus anthracis are centrally located. They form only in the presence of oxygen, thus they are not found in the blood or tissues of affected animals unless same are exposed to free oxygen long enough for spores to form.

The anthrax bacillus stains well with the ordinary aniline dyes and is Gram-positive. Anthrax bacilli in fresh material, when stained with dilute carbol-fuchsin, are frequently found to contain one or more deep-staining granules adherent to the wall of the cell. The spores are not stained by

the ordinary methods but can be stained by special methods described in the section on Stains and Staining. The capsules, likewise, are best brought out by the special capsule staining methods already described.

Cultural Characteristics. Bacillus anthracis is an aerobe but will grow less satisfactorily under anaerobic conditions. It grows best at the usual incubator temperature of 37.5°C. but will develop at temperatures ranging from 12° to 45°C. Outside of this temperature range spores are not formed. Further, cultivation at a high temperature, over a considerable period of time, reduces the virulence of the organism (see Immunization). Bacillus anthracis will grow readily on most of the usual media. It prefers a slightly alkaline or neutral reaction but its growth is not materially interferred with if the medium is slightly acid.

Agar. On agar plates, flat, grayish-white, opaque colonies with irregular borders develop. Examination of the colonies with the microscope will show them to consist of a rather dense, grayish center of compact, wavy, hair-like filaments which, as they spread towards the periphery of the colony, are less compact ("Medusa head" colonies). As the colony becomes older, it appears dry, more granular and the border becomes more irregular. On agar slants a grayish-white, granular, confluent, rather tough growth occurs.

Gelatin. Colonies on gelatin plates are similar to those on agar. The gelatin is slowly liquefied, however, so that the colony is shortly surrounded by a small zone of liquefaction. In gelatin stab cultures growth occurs all along the line of inoculation but is more abundant at the top. Filaments radiate out from the streak giving the whole growth, which has the general shape of an inverted cone, a so-called "inverted pine tree" appearance. As liquefaction occurs a cup forms at the top and gradually proceeds downward, the whole upper stratum shortly becoming liquefied.

Bouillon. Broth is not rendered turbid by Bacillus anthracis. A flocculent growth which sediments to the bottom occurs leaving the broth clear. If the tube is slightly swirled, a light, fluffy growth resembling tufts of cotton arises and slowly settles to the bottom again.

Litmus Milk. Bacillus anthracis slowly acidifies litmus milk. It first coagulates and later peptonizes the casein.

Potato. On potato a luxuriant, grayish-white, dry growth develops. Spore formation occurs early and is well-marked when Bacillus anthracis is grown on this medium.

Peptone Solution. No indol is produced by Bacillus anthracis when grown in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites.

Fermentable Media. The ordinary fermentable media are not changed by Bacillus anthracis. Some strains produce acid from dextrose.

Resistance. The anthrax bacillus, itself, is not very resistant being destroyed by a temperature of 60°C. within 15 minutes. It is likewise easily killed by the ordinary antiseptics in usual dilutions. On the other hand, the anthrax spore is one of the most resistant forms of organisms with which we have to deal. Under certain conditions spores will survive for years without losing their virulence. In soil, in water, hides, etc. the anthrax spore will live for years. Pickling and curing processes will not affect spores in meat thus treated. Dry heat is far less effective for the destruction of the spores of the anthrax bacillus than moist heat. It requires 3 hours exposure to a dry heat temperature of 140°C. to destroy them, whereas a temperature of 100°C. will prove effective in 8 minutes when the spores are in fluid or directly exposed to live steam.

Of the antiseptics bichloride of mercury is most effective and its value can be enhanced by adding 0.50 per cent hydrochloric acid to the solution. While phenol will, in time, destroy the anthrax spore it is not as satisfactory as other disinfectants. Formalin is effective in 1 per cent solution.

Serological Reactions. The complement-fixation and precipitation tests can be utilized in the diagnosis of the disease but ordinarily bacteriological examination and animal inoculation tests are the simplest. In carrying out the complement-fixation test an antigen is made from pieces of the liver or spleen of the suspected case. A small amount of such tissue is macerated with sterile physiological salt solution, filtered through several thicknesses of gauze and titrated against anti-anthrax serum and a control (normal) serum. Because of the danger of infection this test is practically never resorted to.

The precipitation test requires an immune serum known to be of value as a precipitating serum. All anti-anthrax sera are not suitable for such purpose. The test is carried out by boiling a small amount of the affected tissue (spleen, liver or even blood) in 8 parts of physiological saline solution and filtering same through paper or asbestos wool or centrifugalizing. After the filtrate has cooled it is stratified over a little of the precipitating serum in a test tube. A grayish-white, cloudy ring at the point of contact is diagnostic of anthrax. This reaction can also be obtained with culture extracts.

Biological Therapy and Prophylaxis. In 1895 Marchoux and, at about the same time, Sclavo prepared an anti-anthrax serum from sheep and horses immunized against the disease. Since that time anti-anthrax serum has been more or less used in the treatment of anthrax, particularly in man. In

the United States the serum treatment of anthrax in the human family has come into rather wide use during the past ten years. The results from serum in the treatment of the disease, while quite good in human cases, unfortunately, are not as satisfactory with the lower animals. This is undoubtedly due to the fact that the disease in the lower animals is generally a more acute and generalized condition. Some cases in animals, however, are saved by serum so that its use is fully justified where attempts are made to treat affected animals.

Fortunately, anthrax is one of the serious diseases of the lower animals which can be largely controlled through immunization of susceptible animals with properly prepared biological products. The first attempt at immunization against anthrax was made by Toussaint[5] in 1880. He found that fresh blood containing anthrax bacilli could be rendered safe for injection by heating at 55°C. for 10 minutes or by adding 1 per cent phenol to it, and that such material was of value for immunization purposes.

Pasteur's Method of Immunization. The year following Toussaint's report Pasteur demonstrated that the anthrax bacillus could be attenuated by prolonged growth at a high temperature. This finding was the basis for the epoch-making work in anthrax immunization accomplished by Pasteur and his associates Roux and Chamberland.[6] It was found that when a virulent strain of the anthrax organism was grown in broth at a temperature of 42° to 43°C. for 12 days it was reduced in virulence to such an extent that it would prove fatal to mice and guinea pigs but not rabbits. When the growth was continued at such temperature for a total period of 24 days it was found that the culture was attenuated to the point where it was fatal only for mice and not for guinea pigs and rabbits. It was further found that once attenuated by growing at the higher temperature the organism would retain such lowered virulence without fluctuation when subsequently grown at the usual incubation temperature of 37.5°C.

Utilizing cultures thus attenuated Pasteur prepared for immunization purposes two types of vaccine. Fresh bouillon cultures of the strain so attenuated that it would kill mice were made and termed "premier vaccin." Similar cultures were made from the less attenuated strain which would kill guinea pigs but not rabbits and termed "deuxiéme vaccin." With such vaccine Pasteur, in 1881, conducted at Pouilly-le-Fort, before a specially appointed commission, his world-famed anthrax immunization experiment. Fifty sheep were employed, half of such number receiving first the weaker vaccine and 12 days later the stronger vaccine, while the remaining 25

[5] Toussaint, Comptes rend. de l'acad. des sci., xci, 1880.

[6] Pasteur, Chamberland and Roux, Comptes rend. de l'acad. des sci., xcii, 1881.

animals served as controls. Fourteen days after the second injection of vaccine all of the fifty sheep were artificially infected. Within 48 hours the 25 control sheep had succumbed to anthrax. Those which received the vaccine survived.

Pasteur's method of vaccination has been used on a large scale and has been a tremendous factor in the control of anthrax. As a routine procedure the No. 1 vaccine, as prepared commercially, is usually administered in doses of 1 cc. to cattle, horses and mules and in 0.50 cc. amounts to sheep and goats. This is followed in 10 days by the No. 2 vaccine in similar doses. Some firms prepare a special vaccine for sheep. This differs from the regular product in that the No. 2 vaccine is made from a culture about midway between the regular No. 1 and No. 2. vaccine strains in virulence, i.e., such cultures will kill mice and only a small percentage of guinea pigs.

While the Pasteur method of immunization has been a great weapon in the control of anthrax it has certain short-comings. First of all, a small percentage of animals succumbs as a result of the vaccination, especially when exposed to conditions which tend to lower their normal resistance following vaccination. Then the animals must be handled twice. This is, obviously, a big factor with large herds of range animals. Finally, as Pasteur anthrax vaccine is actually a bouillon culture of the organism it frequently becomes inert through autolysis if kept for several months at temperatures favorable for growth. About 10 years ago the writer tested a large number of specimens of Pasteur anthrax vaccine purchased on the open market and found that a considerable percentage of such specimens contained only a few or no living organisms. As it has been well established that dead anthrax bacilli are of practically no value for immunization purposes Pasteur vaccine containing no viable organisms is, obviously, worthless.

The Sobernheim or Simultaneous Method of Immunization. In 1902 Sobernheim[7] recommended a simultaneous method of vaccination against anthrax. For this purpose 5 to 10 cc. (usually 10 cc.) of anti-anthrax serum are injected subcutaneously on one side of the body and followed immediately with an injection on the other side of 0.50 to 1 cc. (usually 1 cc.) of anthrax vaccine corresponding in virulence to that of Pasteur's No. 2 vaccine. This method overcomes several of the short-comings noted with Pasteur's method. The immune serum injected confers an immediate passive immunity which gives some protection at once against natural

[7] Sobernheim, Handbuch der Path. Mikroorg., Kolle & Wassermann, vols. iii and iv, 1904.

infection and also makes it possible to administer vaccine of Pasteur's No. 2 virulence without the previous injection of the No. 1 culture. This of course necessitates but one handling of the animals. The losses from vaccination alone are practically nil.

In the United States, Eichhorn[8] and the author conducted extensive experiments with the simultaneous method of vaccination against anthrax and found that a potent anti-anthrax serum and a *spore vaccine* of the virulence of Pasteur's No. 2 strain gave quite satisfactory results in the control of the disease and that such method possessed several distinct advantages over the Pasteur method. Following this work the simultaneous method of immunization against anthrax became quite popular in the United States and other countries and has been used on a rather large scale.

After this method of immunization came into rather general use in the United States the author paid careful attention to reported results and concluded that while the method possesses many advantages, the degree of immunity conferred was probably not quite sufficient to protect as high a percentage of animals, in badly infected districts, as was hoped for or expected.

Eichhorn[9] has recently expressed a similar conclusion and recommends for use, in districts where anthrax is extensively prevalent in a virulent form, anti-anthrax serum and a vaccine prepared from a culture a little more virulent than that of the Pasteur No. 2 strain, i.e., a culture which will occasionally kill young rabbits as well as mice and guinea pigs. As he points out, some severe reactions and an occasional loss are to be expected from the use of the more virulent vaccine.

Chauveau's Vaccine. Chauveau utilized for immunization purposes a vaccine prepared by a combination of methods for attenuating the anthrax organism. He first grew his cultures at a temperature ranging between 43° and 47°C., then at 37°C. until spores formed, and finally heated them at a temperature of 80° to 84°C.

This method of immunization has not been widely used.

Immunization with Aggressins. In connection with his work on aggressins, Bail reported that he was able to immunize sheep against anthrax by giving them subcutaneously injections of small amounts of sterilized edematous fluid from anthrax cases. The production of immunity is explained by Bail's general theory that the bacteria produce aggressins which are antagonistic to the antibacterial forces of the body. He found that such aggressins are contained in the edematous fluid and when injected without

[8] Eichhorn, B. A. I. Bulletin, No. 340, 1916.

[9] Eichhorn, Jour. A. V. M. A., N.S., vol. xxi, no. 3, p. 284, December, 1925.

living organisms the body reacts and produces anti-aggressins which immunize the animal to subsequent infection with the organism.

The aggressin method of immunization has not been tested out on any considerable scale and has apparently received but little attention. It has recently been taken up by a commercial firm in the United States but little data has been published on results obtained with such product.

For the details of preparing anti-anthrax serum and anthrax vaccine the reader is referred to the section on the Preparation of Veterinary Biological Products.

CHAPTER XXI

Order—Eubacteriales, Family—Bacillaceae (Continued). Organisms of the Genus Clostridium

The genus Clostridium includes the spore-bearing, anaerobic bacteria. The specifications for the genus, as given by the Committee of the Society of American Bacteriologists are:

Anaerobes or microaerophiles, often parasitic. Rods frequently enlarged at sporulation, producing clostridium or plectridium forms.

The pathogenic species of this genus of interest in veterinary medicine are C. chauvei, C. of bradsot, C. oedematis-maligni, C. tetani, C. botulinum and C. luciliae.

Clostridium chauvei (bacillus chauvei, bacillus gangraenae emphysematosae, bacillus of blackleg)

Clostridium chauvei is the causative agent of blackleg, symptomatic anthrax, quarter-ill or quarter-evil, a highly fatal disease of cattle and occasionally other ruminants. Man is not susceptible.

In the early days blackleg was confused with anthrax, the malady being considered merely a particular form of such disease. In the latter part of the eighteenth century Chabert differentiated blackleg from charbon on the basis of clinical and postmortem manifestations and originated the name "charbon symptomatique." Differentiation from an etiological standpoint was not accomplished until 1875. In that year Bollinger[1] published evidence of different causative agents. The following year Feser[2] published the report of his investigations which supported Bollinger's contentions. Final evidence that anthrax and blackleg were separate and distinct diseases was furnished by Arloing, Cornevin and Thomas[3] in 1880. While these latter investigators cleared up the etiology of the disease and shortly afterwards developed a method of immunization against the malady, it remained for Roux[4] (1887) to first cultivate the organism in artificial

[1] Bollinger, Deutsche Zeitschrift f. Tiermed. und Vergleich. Path., i, 1875.

[2] Feser, Zeitschrift fur Prak. Vet. Wissen, iv, 1876.

[3] Arloing, Cornevin and Thomas, Le Charbon symptomatique du boeuf, 2nd ed., Paris, 1887.

[4] Roux, Annales de l'Institut Pasteur, i, 1887.

medium. Later Kitasato[5] (1889) contributed to methods for the cultivation of Clostridium chauvei in pure culture.

Blackleg is principally a disease of young cattle, animals between 6 months and 3 years of age being most susceptible. Calves under six months of age and adult cattle over 3 years old are but rarely affected. The disease occasionally affects sheep, goats and hogs. The affection is one of the so-called "pasture diseases," the causative organism commonly inhabiting the soil in localities where the disease is prevalent.

The malady runs an acute and usually fatal course. It is characterized by fever and crepitating swellings in the musculature, particularly that of the hind or fore quarters. On postmortem examination the most important changes are noted in the muscles and the subcutaneous tissue in the vicinity of the affected area. The subcutaneous tissue is usually infiltrated with a yellowish or blood-tinged, gelatinous exudate, containing hemorrhagic areas of varying size and a considerable number of gas bubbles. The affected musculature appears dark brown or dark red, in some places yellowish. Then black areas or streaks are noted on cut section. Parts of the muscle tissue appear moist, exuding on pressure a dark red fluid containing gas bubbles. Other parts of the musculature, particularly the deeper portions, appear dry and sponge-like due to the action of the gas produced. The affected tissues give off a characteristic, sweetish odor.

Clostridium chauvei can be demonstrated in large numbers in the affected musculature, subcutaneous tissue, fluids and lymph glands of the affected region and usually in the liver. Several hours after the animal's death the organism is present in the blood.

Morphology and Staining Characteristics. Clostridium chauvei is a large, sporulating rod with rounded ends, the average measuring about 0.50 micron in thickness and from 3 to 6 microns in length. Both long rods and shorter, more oval or lemon-shaped forms (clostridium) are seen. The organism is motile by means of peritrichous flagella. The spores are located near the end in some of the rods while in others they are central. Those with end spores appear spoon-shaped.

Unlike the anthrax organism, free oxygen is not necessary for sporulation by Clostridium chauvei. Spores thus form in the affected tissues. Clostridium chauvei usually occurs singly and in pairs, rarely in very short chains.

This organism stains well with the ordinary aniline dyes and is usually Gram-positive. Variable results, however, are obtained with Gram's method of staining, some strains proving Gram-negative.

[5] Kitasato, Zeitschrift für Hygiene und Infektionskrankheiten, vi, 1889.

Cultural Characteristics. Clostridium chauvei is a strict anaerobe. Its optimum temperature requirement is 36° to 38°C. but it will grow a little less rapidly at lower temperatures. Of the various culture media Martin's peptone bouillon, liver broth, chicken broth, Hibler's brain medium and media containing bovine serum and glucose are best suited for its propagation. A meat mash medium consisting of ground beef or chicken in Martin's peptone bouillon or in liver broth is excellent for the growth of Clostridium chauvei. It will grow in media of slightly acid, neutral or slightly alkaline reaction, the latter being preferable. Fluid cultures of Clostridium chauvei give off a butyric-acid odor.

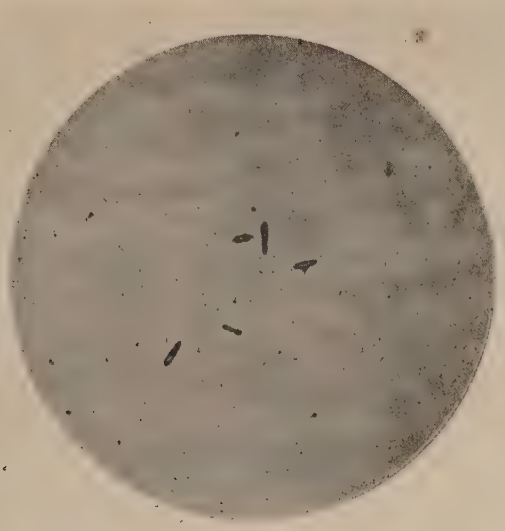


FIG. 44. CLOSTRIDIUM CHAUVEI

Agar. On agar plates, small, slightly irregular, round, semi-opaque, grayish-white colonies with a finely granular, compact center and a less dense peripheral zone develop within 24 to 36 hours. On agar slants an opaque, grayish-white, granular growth occurs.

Gelatin. In gelatin stab cultures a fine, radiating, grayish-white growth occurs followed by liquefaction of the medium. The colonies on gelatin plates are similar to those on agar.

Bouillon. Under proper atmospheric conditions the growth of Clostridium chauvei in bouillon, especially that containing serum, is rapid and

abundant. The medium is first rendered uniformly turbid with evidence of gas production. Subsequently, a grayish-white sediment collects at the bottom of the tube leaving the upper portion of the medium clear.

Litmus Milk. Litmus milk is acidified and coagulated. The coagulation in many instances is imperfect.

Potato. Potato is not very suitable for the growth of Clostridium chauvei, only a very scanty growth occurring.

Peptone Solution. Indol is not formed in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites.

Loeffler's Serum Medium. This is a favorable medium for the growth of Clostridium chauvei. No liquefaction occurs.

Hibler's Brain Medium. Clostridium chauvei grows well on macerated brain medium but does not blacken it as do some of the bacteria of this genus.

Fermentable Media. Clostridium chauvei produces acid and gas from dextrose, lactose, sucrose, maltose, galactose and levulose. Salicin, mannite and dulcite are not fermented.

Toxin Production. When propagated in suitable media Clostridium chauvei produces a toxin which will produce death in guinea pigs, sheep and cattle when injected in sufficient quantity. Toxin production varies greatly according to the medium in which the organism is grown. Further, the production of toxin apparently varies with different strains of the organism.

For the production of toxic culture filtrates the author obtained good results from Martin's peptone bouillon to which calcium carbonate and ground beef had been added. Dextrose-liver-broth with calcium carbonate and ground beef likewise gave good results.

The blackleg toxin loses its potency rather rapidly when exposed to the air.

An antitoxin has been prepared by immunizing animals against the toxin.

Aggressins. Clostridium chauvei is one of the organisms which produces in the tissues a substance which Bail termed "aggressin."

Resistance. In its spore form Clostridium chauvei is an exceedingly resistant organism. It will resist a temperature of 100°C. for several hours. At a temperature of 110°C. six or seven hours are required to destroy the organism. In boiling water an exposure of two hours is necessary to destroy Clostridium chauvei. In dried muscle tissue the spores have been found to retain their virulence for eight or nine years.

While long exposure to high temperatures does not kill the spores it decreases their virulence, probably through destruction of their toxin. This fact has been made use of in the preparation of immunizing products.

Of the chemical disinfectants corrosive sublimate is most effective. A 1:500 solution destroys spores within 10 minutes. Phenol is effective but quite slow in its action. Formalin in 3 per cent solution destroys spores within 15 minutes.

The virulence of Clostridium chauvei is materially increased when agents which retard phagocytosis are introduced with the organism. Thus, foreign material, various other bacteria, lactic acid, etc., if introduced with blackleg organisms which have been attenuated by heat or some other means, will frequently make it possible for such organisms to produce fatal cases of the disease.

Pathogenicity. Of the laboratory animals the guinea pig is most suitable for inoculation purposes. Intramuscular inoculation of virulent material into the hind leg of such animal usually proves fatal in from 18 to 36 hours. Mice are likewise very susceptible. Rabbits and rats are highly resistant. The use of rabbits constitutes a means for the differentiation of blackleg and malignant edema, the rabbit being highly susceptible to the latter disease.

Serological Reactions. Complement-fixation and agglutination tests have been resorted to principally for the identification of cultures of Clostridium chauvei. Such tests, however, apparently are not extensively employed. Cultures of Clostridium chauvei are agglutinated by anti-blackleg serum in dilutions as high as 1:5000.

Biological Therapy and Prophylaxis. Blackleg antitoxin and anti-blackleg serum have been prepared and used in the treatment of the disease. The results, however, have been very discouraging. On the other hand, results from biological products used as prophylactic agents, have been highly satisfactory and have played a tremendous part in the control of blackleg. Quite a variety of methods have been developed and advocated for immunization against blackleg.

Arloing, Cornevin and Thomas were the first to develop a vaccine for the prevention of the disease. Several types of product were prepared but the one which proved most satisfactory was made by grinding up affected muscle tissue, drying it in thin layers at 37°C. and then adding two parts of water to one part of the dried, ground tissue. One-half of such mixture was then heated at a temperature of 100° to 104°C. for seven hours while the other half was heated for the same length of time at a temperature of 90° to 94°C. The treatment consisted of two injections given several days apart. For the first injection the vaccine heated at the higher temperature was used and this was subsequently followed with an injection of the product heated at the lower temperature.

The above method was later modified by Preisz. He attenuated the ground affected muscle tissue by subjecting same to live steam for six hours. Of such product he administered 0.05 gram as the first dose and twice that amount at the second injection.

Another modification of the Arloing, Cornevin and Thomas method was made by Kitt. He prepared a vaccine from powdered affected muscle tissue attenuating it by subjecting it to six hours' exposure to a temperature of 97°C. obtained with steam. The material was then dried and again powdered. A single injection of such vaccine was found to be sufficient to confer a satisfactory immunity. Kitt's method has been used on a large scale. In the United States it was slightly modified by Nörgaard and has been employed for the prophylactic treatment of millions of animals. Nörgaard's modification consisted in heating the material in a hot air oven containing a pan of water to saturate the air with moisture, at a temperature of 93° to 94°C. for six hours.

Various other methods of immunization against blackleg have been used in different countries with more or less satisfactory results but up until the development of "blackleg aggressin" and "blackleg culture filtrate" Kitt's or some other modification of Arloing, Cornevin and Thomas' method was employed on the largest scale.

Schöbl, in 1912, reported the successful immunization of calves against blackleg by injecting them with aggressin containing products prepared from tissues from blackleg cases. This method of immunization, which is now a great factor in blackleg control, did not come into general use in the United States until Franklin and Haslam[6] took it up in 1915. Following the work and reports of these investigators the manufacture of "blackleg aggressin" was undertaken on a large scale by various of the biological firms of the country. At the present time the product is used extensively with excellent results.

Bacteria-free filtrates of cultures of Clostridium chauvei have, likewise, rather recently, come into common use in blackleg immunization. While satisfactory results are obtained from such filtrates they are not as highly efficacious as the aggressin prepared from affected tissues.

For details of the preparation of the principal blackleg biological products the reader is referred to the section on the Preparation of Veterinary Biological Products.

[6] Franklin and Haslam, Jour. Inf. Dis., vol. xix, no. 3, September, 1916.

CLOSTRIDIUM OF BRADSOT (BACILLUS OF BRADSOT, BACILLUS OF GASTROMYCOSIS OVIS)

For years a disease of sheep known as "bradsot," "braxy" or "gastromycosis ovis," believed to be caused by a clostridium very similar to Clostridium chauvei, has been recognized in various parts of Norway, Denmark, Iceland, Faroe Islands, Scotland and Germany.

Bradsot is a disease of young animals, principally affecting sheep between one and three years of age. It is usually rapidly fatal. The disease is characterized chiefly by an acute, hemorrhagic gastroenteritis frequently with necrotic foci in the stomach and duodenum.

Morphology and Staining Characteristics. The Clostridium of bradsot is somewhat similar to Clostridium chauvei. It is a large, motile, sporulating rod averaging about 0.80 micron broad and 3 to 6 microns long. Centrally located spores are most common but end spores occur. The organism is found singly, in pairs and frequently in long chains. The latter characteristic serves to differentiate it from the blackleg organism. It stains with the usual aniline dyes and is Gram-positive.

Cultural Characteristics. The bradsot clostridium is a strict anaerobe. It grows best at the usual incubator temperature of 37.5°C. but will grow rather luxuriantly at lower temperatures. The presence of glucose aids materially in its propagation in artificial culture media. Media of a slightly alkaline reaction are most suitable. In its other cultural characteristics the organism does not differ materially from Clostridium chauvei. It liquefies gelatin, acidifies and coagulates litmus milk and ferments dextrose, lactose and maltose.

Pathogenicity. Guinea pigs, rabbits, mice, chickens, etc., are susceptible. Intramuscular inoculation produces changes very similar to those of blackleg.

CLOSTRIDIUM OEDEMATIS-MALIGNI (VIBRION SEPTIQUE, BACILLUS OEDEMATIS-MALIGNI)

In 1877 Pasteur[7] isolated an anaerobic, spore-bearing organism from animals which were thought to have died of anthrax. He named this organism "Vibrion septique," believing it to be a vibrio rather than a bacillus. About four years later (1881) Koch and Gaffky[8] isolated an organism which they believed to be the same as that isolated by Pasteur and termed it the "Bacillus of malignant edema."

[7] Pasteur, Bull. de l'Acad. Med., Paris, vi, 1877.

[8] Koch and Gaffky, Mitteilungen des Kaiserlichen Gesundheitsamtes, i, Berlin, 1881.

Malignant edema has become well known to the veterinary profession and there is no question as to its importance. However, there is considerable difference of opinion as to whether or not the organism first isolated by Pasteur (Vibron septique) and that subsequently described by Koch (Bacillus of malignant edema) are identical. A number of investigators contend that the two organisms are not the same. They consider that Pasteur's "Vibrion septique" is the causative factor of the malady commonly known as "malignant edema" and that the organism isolated by Koch is probably identical with the organism we today term "Clostridium sporogenes," an anaerobe which possesses practically no pathogenic properties for domestic animals. The contention that the two organisms are not the same is based chiefly on the indication that the organism which Koch and Gaffky isolated was markedly proteolytic whereas Pasteur's organism was not. While no definite proof exists to prove the contention, it appears likely that the organism isolated by Koch and Gaffky was actually Clostridium sporogenes.

As employed in this text, the term "Clostridium oedematis-maligni" is used as a synonym for Pasteur's "Vibrion septique." The question as to whether or not it is likewise a synonym for Koch and Gaffky's original "Bacillus edematis maligni" cannot be conclusively settled from the information at hand.

Clostridium oedematis-maligni is widely distributed in nature, it being constantly present in the superficial layers of the soil in various parts of the world. It usually enters the body through wounds or abrasions. Infection frequently follows castration, sheep shearing, docking, etc., but the organism may occasionally gain entrance to the tissues through other channels. It is pathogenic for man and various lower animals.

Malignant edema in the lower animals is an acute, febrile disease, usually terminating fatally. The horse is the most susceptible of the domestic animals. Sheep are probably next in order of susceptibility, cattle are less often affected and hogs but rarely. Dogs and cats are likewise rarely affected. In cows the disease occasionally follows difficult parturition, especially when the animal has received unskilled help with no regard for aseptic precautions.

Malignant edema is characterized chiefly by a hot, painful, crepitating swelling, usually of considerable size, at the point of infection. This later becomes less painful, cooler and more edematous. On post-mortem examination a yellowish or reddish, serous or gelatinous exudate, containing a large number of gas bubbles, is found in the subcutaneous tissues of the affected region. Hemorrhages of various size are found scattered through

the subcutaneous tissue. The involved musculature is usually of a dark red color and quite brittle. The regional lymph glands are usually markedly swollen and hemorrhagic. Considerable serous fluid of a reddish tinge is generally present in the abdominal cavity. The liver and kidneys reveal evidence of parenchymatous degeneration. Ordinarily the spleen is not appreciably swollen or changed. This latter fact helps to differentiate the disease from anthrax. The lungs show evidence of congestion and edema. The blood is dark and incompletely coagulated. When the infection has followed parturition the uterus and surrounding tissues are edematously

FIG. 45. CLOSTRIDIUM OEDEMATIS-MALIGNI

infiltrated and a thick, brownish-red, foul-smelling discharge exudes from the vagina. Clostridium oedematis-maligni can be readily demonstrated in the edematous fluid and tissues of the affected area and shortly after death in the blood, various organs, etc.

Morphology and Staining Characteristics. Clostridium oedematis-maligni is a large, sporulating rod, quite similar in appearance to the blackleg organism. In size it is usually about 0.80 to 1.00 micron wide and from 2 to 10 or 12 microns long. It varies between rather wide limits in length. The organism is motile by means of peritrichous flagella. The spore of the malignant edema organism is usually centrally located but in some of the rods it

is found near the end. The clostridium occurs singly, in pairs and in long filaments. Such long filaments are particularly common in the livers of guinea pigs succumbing to inoculations of material containing Clostridium oedematis-maligni. This finding is of value in differentiating the organism of malignant edema from Clostridium chauvei.

Clostridium oedematis-maligni stains well with the ordinary aniline dyes and is Gram-positive. However, in the latter respect it behaves like the blackleg organism in that it sometimes appears Gram-negative, especially if decolorization is continued over too long a period.

Cultural Characteristics. Clostridium oedematis-maligni is a strict anaerobe. Under suitable atmospheric conditions it can be cultivated on most of the ordinary culture media but grows best in media containing albuminous material and carbohydrates. It grows most rapidly at the usual incubator temperature but will propagate satisfactorily at room temperature. Media of a slightly alkaline reaction are preferable but the organism is not very fickle in this respect, growing well in slightly acid or neutral media.

Agar. On agar plates, small, dull, slightly irregular, slightly opaque, grayish-white colonies develop. These are made up of a fine, irregular net-work of filaments spreading out from a rather dense center towards the periphery. The growth on agar slants is of a semi-opaque, grayish-white character.

Gelatin. On gelatin plates the colonies are similar to those described as occurring on agar. Liquefaction of the medium occurs after several days. In gelatin stab cultures grayish-white, lentil-shaped colonies develop along the line of the stab in the depths of the medium. This is followed by liquefaction of the medium.

Bouillon. Bouillon is at first rendered uniformly cloudy. Within several days, however, a grayish-white, flaky sediment forms leaving the bouillon above clear.

Litmus Milk. Litmus milk is acidified and partially coagulated after several days. Gas is produced.

Potato. Potato is not a very suitable medium for the cultivation of Clostridium oedematis-maligni. Only a very scanty growth occurs.

Loeffler's Serum Media. Growth readily develops on this medium. It is not liquefied.

Hibler's Brain Medium. Clostridium oedematis-maligni grows well in macerated brain medium without blackening it.

Fermentable Media. Clostridium oedematis-maligni grows well in media containing dextrose, maltose, lactose, galactose, levulose and salicin, fermenting these substances with the production of acid and gas.

Toxin Production. Clostridium oedematis-maligni produces a rather potent toxin when grown in suitable media. Bouillon containing 0.20 per cent dextrose and 10 per cent horse serum is very favorable for toxin production. Filtrates of 48-hour cultures of the organism usually kill guinea pigs and rabbits in doses of 0.50 cc. to 1.00 cc. when injected intravenously. The toxin is effective within a few minutes after injection. An antitoxin can be prepared by injecting horses or sheep with gradually increasing amounts of the toxin.

Resistance. Like the majority of the spore-bearing organisms, Clostridium oedematis-maligni is very resistant. In fluid the spores are destroyed by boiling for 20 minutes. Dry heat of 125°C. destroys the spores in 10 minutes. Bichloride of mercury 1:500 to 1:1000, 5 per cent phenol, $3\frac{1}{2}$ per cent liquor cresolis compound, etc., are effective when given plenty of time to act.

Pathogenicity. Guinea pigs, rabbits, chickens, mice, etc., are very susceptible to injections of material containing the malignant edema organism. The rabbit is best suited for inoculation tests having as their purpose the differentiation of blackleg and malignant edema organisms. As mentioned in connection with Clostridium chauvei, the rabbit possesses considerable resistance to infection with such organism but is readily susceptible to Clostridium oedematis-maligni.

Serological Reactions. Agglutination and agglutinin absorption tests are of value in identifying cultures of Clostridium oedematis-maligni.

Biological Therapy and Prophylaxis. Anti-sera and anti-toxin and vaccines prepared along the lines of blackleg vaccine, culture filtrates, etc., have been prepared for the treatment and prevention of malignant edema. In veterinary medicine, however, these products have been only infrequently used. In man anti-sera was used rather extensively during the world war for the prevention of malignant edema following war wounds.

CLOSTRIDIUM TETANI (BACILLUS TETANI)

Tetanus or "lockjaw," caused by Clostridium tetani, is a well-known disease in both veterinary and human medicine. It has been known for ages and up until the time the etiology of the disease was worked out and tetanus antitoxin developed, it was a much feared and frequently dreaded sequel to various types of wounds and injuries.

In 1884 Carle and Rattone[9] produced tetanus in rabbits by inoculating them with material from an infected acne pustule of a person succumbing

[9] Carle and Rattone, Giorm. d. R. Acad. Torino, 1884.

to "lockjaw." This constituted the first definite evidence of the infectious nature of the malady. During the same year (1884) Nicolaier[10] produced the disease in rabbits, guinea pigs and mice through the inoculation of garden soil. This latter investigator described the causative agent of the disease found at the point of inoculation but he did not succeed in cultivating it in pure culture. In 1889 Kitasato,[11] employing anaerobic cultural methods, cultivated Clostridium tetani in pure culture and produced the specific disease in susceptible animals by the inoculation of such cultures. This classical work of Kitasato's not only settled the etiology of tetanus but marked the beginning of anaerobic methods in bacteriology, thus opening up this important group of organisms.

Of the domestic animals the horse is the most susceptible to tetanus. Sheep and goats are probably next in order of susceptibility. Cattle are only slightly susceptible and dogs and cats are only very occasionally affected. Man is highly susceptible. The disease frequently follows nail punctures, castration, difficult parturition, docking, shearing and various injuries in which the wounds become contaminated with soil.

Clostridium tetani is widely distributed in nature. It is a very common inhabitant of the soil, particularly that which has been contaminated with horse manure. It is frequently noted that the soil of certain premises or localities contains the tetanus organism in great abundance whereas in the soil in other places it is relatively less common. Thus, on some stock farms it has been observed that tetanus in horses will almost invariably follow castration or injuries unless antitoxin is administered as a prophylactic. On the other hand, in certain other sections the disease is rare even though best precautions are not taken in caring for wounds. The organism is a common normal inhabitant of the intestinal tract of the horse and other herbivorous animals.

Tetanus is a wound-infection disease characterized by spasmodic contractions of the voluntary muscles. It is a true intoxication due to the soluble toxin of the tetanus organism. In the horse the principal symptoms characterizing the disease are general stiffness, rigidity of the muscles, particularly those of the head and neck, immobility of the ears, slow mastication and difficult swallowing. These early symptoms are soon followed by muscular spasm which usually begins in the musculature of the head and neck regions and gradually spreads posteriorly. The nictitans membrane, when the animal is startled or the head abruptly raised, is drawn over the cornea by muscular contraction and returns to its proper position very slowly. As

[10] Nicolaier, Deutsche Med. Woch., x, 1884.

[11] Kitasato, Deutsche Med. Woch., xxi, 1889 and Zeitsch. für Hyg. und Infek., vii, 1889.

the disease advances the animal assumes a "straddling" position, head and neck extended, musculature rigid, skin tense, nostrils dilated, jaws immobile. Sudden disturbance, such as clapping the hands, will ordinarily throw the animal into a severe spasmodic attack frequently causing it to fall to the ground. Respiration is shallow and rapid. The visible mucous membranes are frequently cyanotic. There are no characteristic postmortem changes in tetanus.

It should be noted that very young cultures or toxin-free spores when introduced into healthy tissue rarely produce tetanus. Infection is favored where the organisms gain entrance into bruised or otherwise injured tissue and where foreign material is present with Clostridium tetani. Thus, soil, other bacteria, etc., promote infection.

Demonstration of tetanus organisms in animals which have succumbed to the disease is seldom possible. If a lesion is present through which the bacteria entered, Clostridium tetani may be demonstrated in preparations from the same. The organisms remain localized at, or in close proximity to, the point of entrance, there producing their powerful toxin which invades the nerve tissue for which it has a particular affinity, thus producing the disease.

Morphology and Staining Characteristics. Clostridium tetani is a rather slender, sporulating rod with rounded ends, measuring 0.30 to 0.60 micron in thickness and 2 to 5 microns in length. It is motile by means of numerous peritrichous flagella. The spore, which is round or nearly so, is never central but usually situated at the extreme end of the rod giving the organism a characteristic, drum-stick appearance, which is practically diagnostic of the organism. Clostridium tetani usually occurs singly but is occasionally found in short chains of several rods, especially in old cultures. The organism stains well with the ordinary aniline dyes and is Gram-positive. Staining of the spores and flagella can only be accomplished through employment of special methods.

Cultural Characteristics. While Clostridium tetani is an obligatory anaerobe it is not as fastidious in this respect as some other members of the Clostridium genus. It will usually grow in the depths of deep tubes or flasks of culture media without a seal if the medium contains tissue in the bottom of the container or if oxygen consuming bacteria are present in the same culture. The tetanus organism grows best at temperatures of 37° to 38°C. but will propagate less rapidly at temperatures as low as 20°C. Media of slightly alkaline reaction are most suitable for the growth of Clostridium tetani but it will grow satisfactorily on media of a neutral reaction and a little less so in the presence of small amounts of acid. The organism can

be cultivated on most of the ordinary culture media but its growth is favored when glucose or maltose is added to the media. The addition of blood serum likewise favors its growth. The meat mash medium commonly employed for the cultivation of anaerobes is very suitable.

Agar. Colonies on agar plates, grown under anaerobic conditions at a temperature of 37.5°C., usually are not visible until after 48 hours. At first they appear as small, somewhat transparent, slightly raised structures with projections radiating out from their central portion. As they become older the colonies appear grayish-white, show an opaque center surrounded

FIG. 46. CLOSTRIDIUM TETANI

by a net-work of radiating threads, and altogether have a somewhat granular or ground glass appearance. In many ways the colonies resemble those of Bacillus subtilis. Stab cultures in agar (or better glucose agar) result in the development of small colonies along the line of stab with a fine growth radiating out from the streak giving the culture a "pine-tree" appearance.

Gelatin. The colonies on gelatin plates are similar to those on agar. Slow liquefaction of the medium results. In gelatin stabs a fine, arborescent growth occurs, followed by slow liquefaction of the medium.

Bouillon. The growth of Clostridium tetani in bouillon renders the medium slightly turbid.

Litmus Milk. Litmus milk is not acidified or coagulated by Clostridium tetani.

Potato. Growth of Clostridium tetani on potato is very scanty and almost invisible.

Loeffler's Serum Medium. Coagulated serum medium is slowly liquefied by Clostridium tetani.

Hibler's Brain Medium. Clostridium tetani, grown on macerated brain medium, slowly blackens the same.

Fermentable Media. Clostridium tetani does not produce acid or gas in the carbohydrate media commonly employed in bacteriology. A peculiar, disagreeable odor is given off by fluid cultures of Clostridium tetani.

Toxin Produced. The most important characteristic of the tetanus organism is its ability to produce a highly potent, soluble toxin, one of the most powerful poisons known. An average-sized mouse is killed by as small an amount as 0.000,005 gram of standard dry toxin. This toxin when inoculated into animals requires an incubation period before symptoms develop. Such period of incubation varies somewhat with the amount of toxin injected but cannot be shortened to less than 8 or 9 hours even when large quantities of the poison are administered. Where toxin is injected into guinea pigs in amounts a trifle in excess of the minimal lethal dose, symptoms develop in about 76 hours.

The method by which the toxin of Clostridium tetani produces its characteristic disease has been the subject of considerable study and experimentation. The poison has a particular affinity for nerve tissue and it is generally believed that it reaches the central nervous system from the point of production by way of the motor nerve trunks. Meyer and Ransom,[12] who conducted extensive investigations with tetanus toxin, concluded that motor nerves constituted the only paths by which the toxin could reach the central nervous system. Even when injected directly into the blood stream the toxin apparently reaches the central nervous system by way of the motor nerve tracts rather than (as might be supposed) by the blood vessels supplying the central nervous system. In such cases, general tetanus occurs, the various groups of musculature becoming affected simultaneously.

Tetanus toxin is effective when injected subcutaneously, intramuscularly, intraperitoneally, intravenously and intracerebrally. Oral administration of tetanus toxin is without effect.

Toxin production can be readily demonstrated in a number of media but a freshly prepared, slightly alkaline, beef or veal infusion broth containing 1 per cent glucose is well suited for such purpose. Maximum toxin production is reached after about 15 days incubation at 37.5°C.

Tetanus toxin is not very stable or resistant. It is sensitive to light, heat and oxygen. Direct sunlight will destroy it in a day or two. When in a fluid state a temperature of 68°C. will render it inert in 5 minutes. In a dry

[12] Meyer and Ransom, Arch. f. Exp. Pharm. und Path., xlix,

state it will resist a temperature of 100°C. for a short period of time. Exposure to a temperature of 165°C. for 15 minutes destroys it. Kept in a dry state, protected from moisture and light, tetanus toxin retains its potency for years. It is very susceptible to the action of acids and alkali.

The tetanus toxin is readily soluble in water but not in alcohol, ether or chloroform.

A very potent antitoxin can be prepared by immunizing animals with increasing doses of the tetanus toxin. Such antitoxin is highly efficacious as a prophylactic agent. Unfortunately, however, it is far less efficient when administered in the treatment of the disease after symptoms have developed. This is undoubtedly due to the fact that once the toxin has combined with the nerve tissue its neutralization with antitoxin is extremely difficult because of the inability of nerve tissue to readily absorb large amounts of the antitoxin injected. In the treatment of tetanus, simultaneous intraspinal and intravenous injections of large quantities of antitoxin should be given.

For details concerning the preparation of tetanus toxin and antitoxin and the standardization of same the reader is referred to the section on the Preparation of Veterinary Biological Products.

Tetanolysin. In addition to the production of a powerful, soluble toxin, Clostridium tetani produces a hemolysin which has been termed "tetanolysin." This product is capable of hemolizing the blood cells of various species of animals. Such hemolysin, however, is of no particular importance in the pathology of tetanus.

Resistance. Clostridium tetani is quite a resistant organism when its spore is considered. The vegetative form is destroyed with little difficulty. The tetanus spore will frequently resist live steam for 15 minutes to one hour or more. Live steam at a temperature of 115°C. destroys it within 5 minutes. Bichloride of mercury in a 1:500 dilution will destroy the spore only after long exposure (several hours). Phenol requires a much longer time. The action of the various chemical disinfectants can be markedly enhanced if 1 per cent hydrochloric acid is added.

The tenacity of the tetanus toxin has already been discussed.

Pathogenicity. The guinea pig, mouse and rabbit are very susceptible to tetanus. The rat and various fowls are highly refractory.

Serological Reactions. Agglutinins can be produced for Clostridium tetani through animal inoculation. The sera of such animals can be utilized in agglutination tests for the identification of the tetanus organism but such test is not ordinarily resorted to because of the relative ease with which Clostridium tetani can be recognized bacteriologically.

Types of Clostridium tetani. Based on agglutinin absorption tests, several investigators, notably Tulloch,[13] have recognized a number of *types* of Clostridium tetani. While there appears to be variation in pathogenicity with different types, the antitoxin of one will neutralize the toxin of the others.

Biological Therapy and Prophylaxis. As already indicated a potent antitoxin can be prepared against the tetanus toxin. Such antitoxin is of tremendous value as a prophylactic agent even if administered some hours after infection, but once symptoms have developed the efficacy of the antitoxin is very limited.

CLOSTRIDIUM BOTULINUM (BACILLUS BOTULINUS)

In 1896 Van Ermengem[14] reported the isolation of a spore-bearing, toxin-forming anaerobe from ham which had caused severe cases of "food-poisoning" in a number of individuals who had eaten of it. The same organism was also isolated from the gastro-intestinal tract and from the spleen of one of the victims. Van Ermengem made a careful study of the organism and named it "Bacillus botulinus." Confirmation of Van Ermengem's work was soon forthcoming with the result that "botulism" became recognized as an occasional, serious "food intoxication" of man.

In the United States, during the last 8 or 9 years, botulism has attracted considerable attention both in human and veterinary medicine. A series of outbreaks of the disease in man, resulting from the ingestion of cheese, ripe olives, canned asparagus, string beans, spinach, corn, apricots and other canned vegetables and fruit, principally home-canned products, have aroused the interest of the medical profession. From a veterinary standpoint the subject has received a great deal of attention largely as a result of the work of Graham[15] and his associates who reported that a number of the outbreaks of so-called "forage-poisoning" or cerebro-spinal meningitis of horses were due to the toxin of Clostridium botulinum. Further, it has been shown that the toxin of one type of Clostridium botulinum causes cases of "limber-neck" in chickens.

Outbreaks of forage-poisoning among horses in the United States have been of frequent occurrence. In some of the outbreaks the losses have been tremendous. In consequence the disease has been the subject of considerable study and investigation. The similarity of the symptoms of equine forage-poisoning to those of botulism in man has been recognized for some

[13] Tulloch, Jour. Hygiene, vol. xviii, 103, 1919.

[14] Van Ermengem, Cent. für Bakt., xix, 1896.

[15] Graham, Brueckner and Pontius, Ky. Agric. Exp. Sta., Bull. No. 207, June, 1917.

time, Leonard Pearson first calling attention to same years ago. Thus, when Graham published his report of the isolation of Clostridium botulinum from oat-hay which was believed to have given rise to cases of equine forage-poisoning, it looked as if the etiology of this malady had finally been determined. Up until the time of Graham's work the prevailing opinion was that cerebro-spinal meningitis was probably due to one or more types of fungi, although various other organisms including streptococci, diplococci, filterable viruses, etc., had been mentioned in connection with the disease.

Unfortunately, evidence accumulated since Graham's first report tends to indicate that, while some cases of forage-poisoning may be due to the toxin of Clostridium botulinum, such agent is by no means invariably the etiological factor. Thus, as the matter now stands, forage-poisoning must be considered a group term with Clostridium botulinum recognized as capable of producing only one type of the disease. At present there are no definite figures to indicate just how large a percentage of cases of cerebro-spinal meningitis which occur in the United States are actually cases of botulism.

There are two definite types of Clostridium botulinum, an "A" type and "B" type. Bengston[16] isolated a somewhat closely related organism, which has been considered by some authorities as a "C" type of Clostridium botulinum but is classified in this work as "Clostridium luciliae" (Bengston), following the classification of the Committee of the Society of American Bacteriologists.

The "A" and "B" types of Clostridium botulinum are impossible of definite differentiation culturally. They are, however, readily distinguished by immunological tests on guinea pigs or other animals, the antitoxin of one type not protecting against the toxin of the other. Further, chickens are readily susceptible to the toxin of the type "A" organism, developing "limberneck," but are refractory to the type "B" toxin.

Clostridium botulinum is widely distributed in nature. It is found chiefly in the soil, but has also been demonstrated in the feces of hogs and other animals, in mouldy hay, leaves, damaged fruits, vegetables, etc. In the United States, judging from outbreaks of botulism in man, the "A" variety of Clostridium botulinum appears to be more common in the western section of the country, while in the east the "B" type seems to predominate.

Clostridium botulinum, in itself, is not ordinarily pathogenic but causes disease through infection with its powerful, preformed toxin. Feeding of toxin-free spores, even in enormous amounts, rarely results in disease. In a series of experiments conducted by the author, in which a number of

[16] Bengston, U. S. Pub. Health Rpts., xxxvii, 1922.

guinea pigs were fed large quantities of toxin-free spores following a preliminary dose of ox bile, one guinea pig developed botulism and the organism was recovered from the spleen. This is in agreement with the opinion of several investigators that the administration of massive amounts of toxin-free spores will occasionally give rise to botulism.

Botulism is characterized by a marked disturbance of the central nervous system. The symptoms are not manifested immediately following the ingestion of contaminated food but appear after a short period of incubation

FIG. 47. CLOSTRIDIUM BOTULINUM

of the toxin, the duration of such period varying, to a certain extent, with the amount of toxin received.

Horses, to which the toxin of Clostridium botulinum has been administered, first show dullness, weakness, labored respiration and evidence of impaired vision. This is followed by nervous excitability, paralysis and convulsions. The head is frequently drawn back or to one side by spasmodic contraction of the neck muscles. There is frequently an intermittent trismus with gnashing of the teeth. The animal, when excited, will often throw its head back or to one side, turn in a circle and may fall to the ground. In chickens botulinus toxin gives rise to paralysis with a peculiar weakness of the neck muscles, giving rise to the characteristic "limber-neck."

Postmortem changes in botulism are very limited. An outstanding condition, frequently noted in animals succumbing to the disease, is a markedly congested condition of the blood vessels of the brain and meninges. The organism can only very occasionally be isolated from the gastro-intestinal tract and rarely from the spleen and other organs. In the first place it is the preformed soluble toxin of Clostridium botulinum which is responsible for the disease and only a very minute amount of same is necessary to produce fatal results even in large animals. Thus, a small amount of the toxin may gain entrance into the body with few or no specific organisms. Then, from a standpoint of differential diagnosis, the situation is compli-

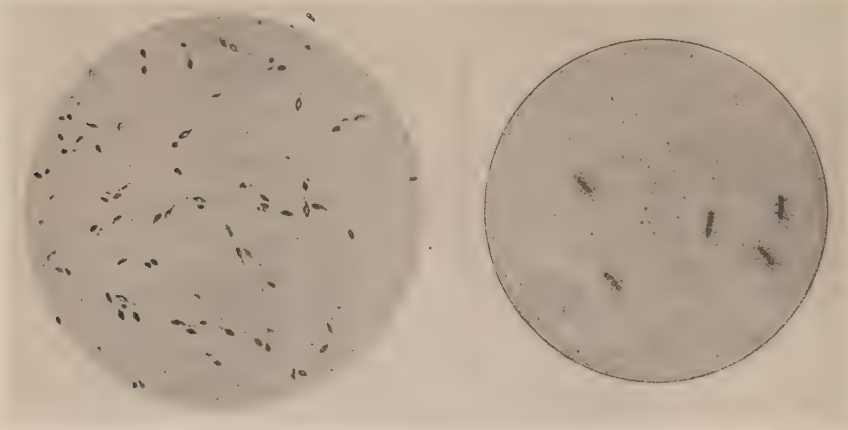


FIG. 48 FIG. 49

FIG. 48. CLOSTRIDIUM BOTULINUM (SPORES STAINED BY ABBOTT'S METHOD)

FIG. 49. CLOSTRIDIUM BOTULINUM SHOWING FLAGELLA

cated by the fact that the organism may be casually present in the intestinal tract without pathological significance.

Morphology and Staining Characteristics. Clostridium botulinum is a large, sporulating, motile rod with rounded ends. It measures 0.90 to 1.2 microns in thickness and from 4 to 6 microns in length. It usually occurs singly but is occasionally found in short chains. The oval spore is usually formed near the end of the rod. The organism stains with the ordinary aniline dyes and is Gram-positive.

Cultural Characteristics. Clostridium botulinum is a strict anaerobe growing best at a temperature ranging from 20° to 28°C. There has been considerable disagreement regarding the temperature requirements of

Clostridium botulinum. Some writers have shared the original view of Van Ermengem that the organism would not grow or produce toxin at body temperature. Contrary to such reports Clostridium botulinum will grow at the usual incubator temperature of 37.5°C. and produce a potent toxin at such temperature. Clostridium botulinum is quite fastidious in its media reaction requirements, a neutral or slightly alkaline reaction being necessary for best results. The organism will grow in most of the usual media but those containing glucose and animal or vegetable tissue are more satisfactory. Dextrose-meat-mash medium is particularly well-suited for its propagation.

Agar. On agar plates (preferably glucose-agar), small, flat, granular, somewhat irregular, grayish colonies develop after 24 hours. As they become older the colonies appear opaque, yellowish-brown in color, and have minute, thorn-like projections around their periphery. In agar (glucose agar) stab cultures a grayish-white growth occurs along the line of stab to within a short distance of the surface. This is followed by considerable gas production and a cracking of the medium. Isolated, deep colonies in agar appear slightly opaque, lenticular or kidney-shaped and may subsequently develop irregular projections.

Gelatin. Colonies on gelatin plates are similar to those on agar. The medium is liquefied. Gelatin stab cultures show a fine growth along the line of stab with evidence of gas formation and later liquefaction.

Bouillon. In broth (preferably glucose broth) a uniform turbidity results accompanied by considerable gas formation. As the culture ages sedimentation occurs leaving the broth clear. Broth and other fluid cultures give off a butyric acid odor.

Litmus Milk. This medium is not very suitable for the propagation of Clostridium botulinum. The litmus is reduced but no coagulation occurs.

Potato. Potato medium is unsuited for the growth of Clostridium botulinum.

Hibler's Brain Medium. This medium is darkened by the growth of the botulism organism.

Coagulated Serum Medium. Clostridium botulinum grows well on Loeffler's serum medium which it liquefies.

Fermentable Media. Clostridium botulinum produces acid and gas from glucose, maltose and sucrose.

Toxin Production. Clostridium botulinum produces a powerful, soluble toxin which is specific for the particular type ("A" or "B") of organism. Very minute amounts of this poison will often produce death. The writer has prepared toxin which would kill guinea pigs in as small an amount as

0.000,1 cc. Some investigators report even smaller minimal lethal doses for guinea pigs.

The toxin of Clostridium botulinum can be readily demonstrated in most all cultures after several days' growth and in fluids from canned food-products in which the organism has grown. For the production of a potent toxin a slightly alkaline, standard beef-infusion broth containing 1 per cent glucose and a small amount of ground beef gives excellent results. Some workers substitute pork-infusion broth for the beef bouillon. The results are not materially different. Such medium, filled in tubes (three-fourths full) or in flasks (nearly up to the neck) and layered with liquid petrolatum, can be used for the cultivation of Clostridium botulinum without special anaerobic apparatus. Just prior to the inoculation of such medium it is advisable to place the tubes or flasks containing same in a boiling water-bath or the Arnold sterilizer to drive off any free oxygen. When the medium has cooled down to about 50°C. it should be inoculated deep, taking care, if a pipette is employed, not to introduce air. The cultures should then be placed in the incubator at once.

Like the tetanus toxin, the toxin of Clostridium botulinum is soluble in water but insoluble in alcohol, chloroform and ether. It is also similar to the tetanus toxin in its affinity for nerve tissue.

The botulism toxin, fortunately, is quite thermolabile. A temperature of 80°C. destroys it in half an hour. It is likewise highly susceptible to oxygen and sunlight. It is quickly destroyed by alkalies but is more resistant to acids.

A potent antitoxin can be prepared against each type of toxin through the immunization of animals.

For the methods of preparing the toxin and antitoxin see section on the Preparation of Veterinary Biological Products.

Resistance. The resistance of the botulism toxin has been given above. Clostridium botulinum and its spores are not as resistant as some of the other sporulating anaerobes, still the spores have been shown to withstand boiling for several hours. Esty and Meyer[17] found the heat resistance of 109 strains of Clostridium botulinum to vary from 3 to 80 minutes when exposed to a temperature of 105°C. They also found that the spores contained in the juices of 17 varieties of canned foods, resisted heat of 100°C. for periods varying from less than 10 minutes to 230 minutes.

The organism will not develop in products containing a rather high salt concentration (8 to 10 per cent), hence, food-stuffs which have been pickled in strong brine are usually safe so far as botulism is concerned.

[17] Esty and Meyer, Jour. of Inf. Diseases, vol. 31, 1922, p. 650.

The ordinary antiseptics, particularly bichloride of mercury, are effective in the usual dilutions but should be given a long period of time in which to act.

Pathogenicity. Toxin-free spores, as already indicated, are not ordinarily pathogenic. Guinea pigs, rabbits and mice are susceptible to the toxin. Chickens are susceptible to the toxin of the "A" type of organism but not to the "B" type.

Serological Reactions. Agglutinins and complement-fixing bodies can be produced with Clostridium botulinum and its toxin. The use of serological tests in cases of botulism, however, offer no help in diagnosis as the intoxication in most cases runs a short, acute course, which, in serious cases, terminates in death before agglutinins or complement-fixing bodies are present in the blood in detectable numbers.

The writer[18] has found the complement-fixation test valuable for the identification of Clostridium botulinum or its toxin when present in culture (pure or contaminated) or in infected, canned food-stuffs. Using antitoxin as positive serum, normal horse serum as negative serum, and the questionable culture, culture-filtrate or preparation of suspected food-stuff as an antigen, a complement-fixation reaction can be obtained if the antigen contains Clostridium botulinum or its toxin. Such test, however, will not differentiate the type of Clostridium botulinum.

The agglutination and agglutinin absorption tests are of value in identifying cultures. Cross agglutination occurs with the types.

For the details of the complement-fixation test the reader is referred to the section on Serology.

Biological Therapy and Prophylaxis. Botulism antitoxin, administered shortly before or immediately following the entrance of toxin into the body, will prevent the development of intoxication provided, always, that the homologous or at least a polyvalent antitoxin is used. As in tetanus, once symptoms of botulism have become manifest, antitoxin is not nearly so efficacious in the treatment of the disease as it is in its prevention. However, in spite of the fact that results are quite discouraging, it is probably the best treatment at our disposal.

In cases of forage-poisoning, believed to be due to the toxin of Clostridium botulinum, the intravenous injection of large quantities of botulism antitoxin (ordinarily polyvalent because the type is not generally known) is fully justified and should be resorted to.

[18] Kelser, Jour. Amer. Pub. Health Asso., May, 1923.

CLOSTRIDIUM LUCILIAE

Bengston[19] in 1922, in examining material submitted for bacteriological study in connection with the occurrence of "limber-neck" in chickens, isolated from the larvae of the green fly "Lucilia caesar," an organism which in some respects is similar to Clostridium botulinum. The organism was found to produce a soluble toxin which is not neutralized by either type "A" or "B" botulism antitoxin. The toxin is weaker than that produced by Clostridium botulinum but will kill guinea pigs, rabbits, monkeys, mice, rats, pigeons and chickens when given in sufficiently large amounts.

Just how much of a factor this organism is in the production of disease in fowls or other animals is at present questionable.

Morphology and Staining Characteristics. As described by Bengston, Clostridium luciliae is a rather large, non-motile, sporulating rod with rounded ends, measuring 0.5 to 0.8 by 3 to 6 microns. It is usually found singly but occasionally is observed in pairs and short chains. Spores are somewhat slow in developing and are found at the end of the rod. The organism stains with the ordinary aniline dyes and is Gram-positive. As the cultures become old they are irregular in their attitude towards Gram's method of staining.

Cultural Characteristics. Clostridium luciliae is a strict anaerobe growing best at the usual incubation temperature of 37.5°C. and less rapidly at room temperature. Media of a slightly alkaline reaction are best suited for its growth.

Agar. In glucose shake agar cultures, small, fluffy colonies develop without gas formation. Hitchen's tenth-per-cent agar is a favorable medium.

Gelatin. The organism grows very slowly in gelatin without liquefying it.

Bouillon. In glucose broth growth is slight with acid formation but no gas production and practically no clouding of the media.

Meat-mash Medium. This is a good medium for the propagation of Clostridium luciliae. Growth occurs with the formation of gas bubbles and results in no appreciable turbidity. Bengston points out that the failure to produce turbidity is characteristic of the organism.

Litmus Milk. Litmus milk is acidified but not coagulated.

Toxin Produced. Cultures several days old are toxic. Bengston found doses of 0.001 cc. or less fatal for mice, rats, guinea pigs and rabbits. A much larger amount (several cubic centimeters) is required to produce symptoms in chickens.

[19] Bengston, U. S. Pub. Health Rpts., xxxvi, January, 1922.

It is noteworthy that rats are quite susceptible to this toxin whereas to the toxin of Clostridium botulinum they are rather refractory.

The toxin of Clostridium luciliae is destroyed by heating at a temperature of 65°C. for 30 minutes.

A potent antitoxin can be prepared through the use of horses, sheep, goats, etc.

Resistance. Clostridium luciliae is destroyed only after an hour's exposure to flowing, live steam. Bichloride of mercury, phenol, formalin, etc., are effective in the usual strength solutions but should be given plenty of time in which to act.

Pathogenicity. As already pointed out mice, rats, guinea pigs, rabbits and monkeys are susceptible to the toxin of Clostridium luciliae. Chickens must be given relatively large amounts if symptoms are to be produced.

Serological Reactions. Agglutination and complement-fixation reactions can be obtained with antitoxin and properly prepared antigens.

CLOSTRIDIUM WELCHII (BACILLUS WELCHII, BACILLUS AEROGENES CAPSULATUS, BACILLUS PHLEGMONIS EMPHYSEMATOSAE, BACILLUS PERFRINGENS)

In 1892 Welch and Nuttall[20] reported the isolation of a large, anaerobic, sporulating rod, which apparently was the cause of rapid development of considerable gas in the body of a man autopsied shortly after death from an aortic aneurysm. They designated the organism "Bacillus aerogenes capsulatus." Subsequently it became generally known as "Bacillus welchii." Since its discovery this anaerobe has been found widely distributed in nature. During the world war it was an important factor in war-wound infections, being present in about 75 per cent of the cases of gas gangrene.

From a veterinary standpoint Clostridium welchii is generally thought to be of no particular importance. The writer is of the opinion, however, that following wound infections in animals this organism may occasionally produce a disease process which could be mistaken for malignant edema. For this reason a brief description of the organism is given.

Morphology and Staining Characteristics. Clostridium welchii is a rather large, non-motile, encapsulated, sporulating rod. The ends of some of the rods appear square-cut while others are rounded. The spores are formed rather reluctantly, frequently being absent in affected tissues and in cultures. The spores are best demonstrated when the organism is grown in an alkaline medium free of carbohydrates but rich in protein. They are

[20] Welch and Nuttall, Johns Hopkins Univ. Bull., iii, 1892.

oval in shape and are usually centrally located but occasionally are found subterminal. The organism occurs singly and in pairs, rarely in chains.

Clostridium welchii stains with the ordinary aniline dyes and is Gram-positive.

Cultural Characteristics. This organism is a strict anaerobe and grows rapidly at a temperature of 37.5°C., less rapidly at lower temperatures. Media of alkaline reaction are best suited for its propagation.

Agar. Agar plate cultures, grown under anaerobic conditions, develop round or oval, opaque, grayish-white colonies. Stab cultures in glucose agar result in abundant growth with the development of large amounts of gas. The large quantity of gas produced by Clostridium welchii in the various media is characteristic of the organism.

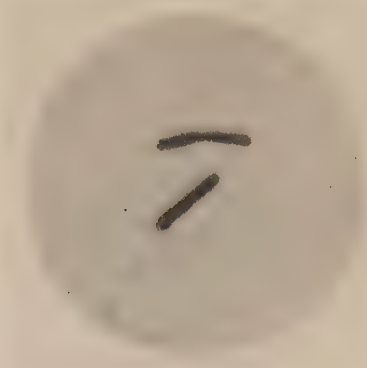


FIG. 50. CLOSTRIDIUM WELCHII SHOWING CAPSULE

Gelatin. Growth in gelatin is poor. The medium is liquefied.

Bouillon. Broth is rendered uniformly turbid. Gas formation is evident.

Litmus Milk. The growth of Clostridium welchii in milk is characteristic. It acidifies and coagulates the milk and causes a marked breaking up of parts of the coagulum as a result of energetic gas formation. This action in milk has been referred to as "stormy fermentation." Cultures give off a butyric-acid odor.

Coagulated Serum Medium. Loeffler's serum medium is not liquefied by Clostridium welchii. This organism is highly saccharolytic but rarely proteolytic. It is never proteolytic when carbohydrates are present.

Hibler's Brain Medium. This medium is not blackened by Clostridium welchii.

Fermentable Media. Clostridium welchii produces acid and gas from glucose, lactose, maltose, levulose, galactose, sucrose, inulin and glycerol.

Toxin Produced. Clostridium welchii produces a soluble toxin. Different strains vary considerably in toxin-producing qualities. A potent antitoxin can be produced.

Types of Clostridium welchii. Four different types of Clostridium welchii have been distinguished. The Type I organism is the common form and is the one described above. Type II differs from the common type in its inability to ferment inulin. Type III differs from Type I in its inability to ferment glycerol. Type IV differs from the common type in that it does not ferment either inulin or glycerol.

Pathogenicity. This organism is not very pathogenic when injected alone into healthy tissue. It is frequently pathogenic, however, for guinea pigs, rabbits and mice when inoculated intramuscularly.

Resistance. A temperature of 60°C. destroys the vegetative form of Clostridium welchii within 15 minutes. The spores, however, are only destroyed after 20 minutes exposure to a temperature of 100°C. The various antiseptics are ordinarily effective in the usual strength solutions within one-half hour.

Serological Reactions. Agglutination and agglutinin absorption tests can be utilized for the identification of cultures of Clostridium welchii.

Biological Therapy and Prophylaxis. An antitoxin is of value in prophylaxis, less so for treatment of cases of Clostridium welchii infection.

CLOSTRIDIUM HEMOLYTICUS BOVIS

A disease of cattle and sheep, known as "ictero-hemoglobinuria," and in lay terms as "red water disease" or "hemorrhagic disease," has, since about 1916, been rather frequently reported from several of the western states, particularly California and Nevada. Meyer,[21] Mack and Records,[22] Records and Vawter,[23] and others have published results of investigations of the disease.

The malady runs a rapid course terminating fatally within 24 to 36 hours. It is characterized by a pronounced hemoglobinuria and occasionally intestinal hemorrhage giving rise to a bloody discharge from the anus. Postmortem examination demonstrates numerous hemorrhages of the subcutis and mucous and serous membranes. Any urine present in the bladder

[21] Meyer, K. F., Jour. A. V. M. A., N.S., i, no. 5, 1916.
[22] Mack and Records, Jour. A. V. M. A., N.S., v, no. 2, 1917.
[23] Records and Vawter, Jour. A. V. M. A., N.S., xiii, no. 2, 1921.

contains blood. In the liver a change reported as constant is a single, large, hemorrhagic infarct.

Up until the middle of 1925 the causative factor of bovine ictero-hemoglobinuria was unknown. In July of that year Vawter and Records[24] presented the results of a careful study of the disease from the standpoint of etiology and have apparently proved that the malady is caused by an anaerobic, sporulating rod which they designated "Clostridium hemolyticus bovis."

Morphology and Staining Characteristics. According to Vawter and Records, Clostridium hemolyticus bovis is a large, sporulating rod with rounded ends. It is a little more than 1 micron in width and varies from 3 to 5.6 microns in length. The organism is motile by means of peritrichous flagella. The spores are either terminal or subterminal. It occurs singly, in pairs and occasionally in short chains. The organism apparently stains satisfactorily with the ordinary aniline dyes and is Gram-negative. However, as regards its behavior towards Gram's method of staining, Vawter and Records indicate that there was some irregularity, depending upon the particular method used and the age of the culture.

Cultural Characteristics. Concerning the cultural characteristics of this organism we quote Vawter and Records:

> Anaerobic conditions must be maintained for growth. Peptic-digest broth plus raw rabbit-liver, sealed with petrolatum paraffin, produces a very rapid, luxuriant growth, causing incomplete clouding in 8 to 12 hours of incubation at 37°C. Varying amounts of gas with a cheesy odor are produced, usually enough to raise the seal 4 cm. and occasionally blow the cotton plug.
>
> Autoagglutination begins as early as the twelfth hour of incubation and continues until clearing of the broth takes place in 36 to 96 hours. Visability is not maintained longer than a few days in sealed cultures.
>
> Growth in unsealed, tall tubes, 200 by 25 mm., of the same medium, is similar to that in sealed tubes, except that complete clouding of the broth does not usually occur until the twelfth hour. Active growth persists longer and autoagglutination does not occur until after 72 to 96 hours.
>
> Beef-heart, peptic digest liver broth, unsealed, produces a slower and less luxuriant growth, attaining the maximum in 24 to 36 hours. Moderate gas production occurs, but often is not noticed unless the deeper meat particles are disturbed. Autoagglutination of the bacilli in fluffy masses around the meat particles in the bottom of the tube occurs after 36 to 48 hours. Slight reddening of the meat particles occurs in cultures as early as the twenty-fourth hour but never becomes prominent; blackening does not occur. The formation of amino-acid crystals has never been observed in meat cultures regardless of age. Proteolytic action does not occur in cultures incubated as long as three months.
>
> Viability is maintained for long periods in beef-heart, peptic-digest broth.

[24] Vawter and Records, Jour. A. V. M. A., N.S., xxi, no. 4, 1926.

Two per cent agar slants, made up either from ordinary meat broth or peptic-digest broth, enriched by the addition of 5 to 7 per cent, sterile, defibrinated, rabbit blood, incubated anaerobically by the sodium hydroxide-pyrogallic acid method of Hall, produce a light surface growth in 24 hours. Hemolysis of blood-agar is diffuse and complete after 24 hours of incubation.

The deep-agar method of Hesse has been used in obtaining pure cultures and for comparative study of colony types produced by various strains. Two per cent agar in peptic-digest broth (pH 7.2) has been found superior to plain, beef-broth agar. Agar is unsuitable for use two days after sterilization, vigorous boiling and agitation of the agar tubes not reducing oxygen concentration to a point tolerated by this anaerobe.

Colonies appear in deep agar after 18 to 24 hours of incubation. They are at first lenticular and homogenous, but rapidly develop an eccentric fluff on both surfaces. In 6 to 8 hours the colonies appear as dense, woolly masses, with short, peripheral filaments. They vary from 0.25 to 0.5 mm. in diameter, according to the number in the tube. Gas is rarely if ever produced, even in heavily seeded, deep-agar cultures.

The organism liquefies gelatin, coagulates and acidifies milk and produces acid and gas in the presence of dextrose, levulose and galactose.

Toxin Produced. Clostridium hemolyticus bovis produces a toxin which is apparently quite unstable. An antiserum has been produced by Vawter and Records which they believe to be of definite value in the treatment and prevention of the disease.

Pathogenicity. Guinea pigs and rabbits are susceptible, death occurring within 16 to 24 hours subsequent to inoculation with toxic cultures.

CHAPTER XXII

Order—Actinomycetales, Family—Actinomycetaceae. Organisms of the Genus Actinobacillus

In most of the older systems of classification of bacteria there is recognized a group of so-called "higher bacteria," made up of filamentous organisms characterized by branching and, in some instances, by the formation of mycelia and conidia. Such higher bacteria have been considered as occupying an intermediate position between the true bacteria and the moulds or fungi. In the system of classification adopted by the Society of American Bacteriologists these higher forms are included in several orders under the class Schizomycetes. One of such orders—Actinomycetales, is of particular importance in veterinary medicine. Two families make up the order, namely, Actinomycetaceae, and Mycobacteriaceae.

The family Actinomycetaceae at present consists of 4 genera, "Actinobacillus, Leptotrichia, Actinomyces, and Ersipelothrix. Of these, all are of interest in veterinary medicine except the genus Leptotrichia.

The description of the genus Actinobacillus, as given by the Committee of the Society of American Bacteriologists, is:

> Filament formation; resembling streptobacilli. In lesions no mycelium formed, but at peripheries finger-shaped, branched cells are visible. Gram-negative. Not acid fast.

At the present time there is but one species in this genus and that is Actinobacillus lignièresi, an organism of interest in veterinary rather than human medicine.

ACTINOBACILLUS LIGNIÈRESI

In 1902 Lignières and Spitz,[1] working in Argentina, described a malady of cattle which in many ways resembled actinomycosis. The same disease has also been reported from France, Germany and Canada.

The malady is characterized by cold abscesses of the subcutaneous tissue, particularly that of the throat region. Less frequently such abscesses occur in the region of the parotid gland, at the base of the ear and over the superior maxillary bone. In some cases the tongue is involved, the condition being very similar to the "wooden tongue" of actinomycosis. Occa-

[1] Lignieres and Spitz, Rev. Soc. Med. Arg., x, 1902.

sionally lesions are produced in the pharynx, lungs and udder. Various other parts of the body may be involved along with the regional lymph glands. The bony structures are rarely affected.

Morphology and Staining Characteristics. In the thick, pasty pus from the characteristic abscesses there are found small, grayish-white granules. Microscopically these consist of rosettes of radiating clubs without the presence of a mycelium. Preparations from cultures on solid media reveal minute rods hardly more than 1.5 microns in length. Preparations from bouillon cultures show long chains or filaments. Thus, the organism differs from Actinomyces bovis principally in its growth on artificial culture media.

Actinobacillus lignièresi stains fairly well with the usual aniline dyes, particularly carbol-fuchsin, and is Gram-negative.

Cultural Characteristics. Actinobacillus lignièresi is an aerobe and facultative anaerobe. It grows well at the usual incubator temperature of 37.5°C. The addition of blood serum to media favors its growth. Media of slightly alkaline reaction are best suited for its propagation.

Agar. If pus containing granules is triturated in a sterile mortar and sown on agar plates, small, translucent, bluish colonies develop within 24 hours.

Gelatin. This organism grows feebly in gelatin and does not liquefy it.

Bouillon. Broth to which serum has been added is rendered turbid and a light pellicle forms.

Litmus Milk. Litmus milk is a good medium for the growth of Actinobacillus lignièresi. It acidifies but does not coagulate the milk.

Potato. A very scanty, slightly yellowish growth occurs on potato.

Peptone Solution. Indol is formed in Dunham's peptone solution to which serum has been added to favor growth.

Nitrate Solution. Nitrates are not reduced to nitrites.

Fermentable Media. Acid is produced in media containing glucose, lactose and maltose.

Toxin. Fluid cultures of Actinobacillus lignièresi contain a toxic substance which will produce fever and nervous symptoms in calves.

Resistance. Actinobacillus lignièresi is not a very resistant organism. A temperature of 60°C. destroys it within 15 m . Drying kills it in a short period of time.

Pathogenicity. Intraperitoneal injection of cultures or pus into guinea pigs produces a peritonitis. Colonies of the organism are present in the peritoneal fluid of such animals. Rabbits and mice are not suitable for inoculation purposes.

CHAPTER XXIII

Order—Actinomycetales, Family—Actinomycetaceae (Continued). Organisms of the Genus Actinomyces

The description of the genus Actinomyces, as given by the Committee of the Society of American Bacteriologists, is:

Organisms growing in form of a much-branched mycelium, which may break up into segments that function as conidia. Sometimes parasitic, with clubbed ends of radiating threads conspicuous in lesions in the animal body. Some species are microaerophilic or anaerobic. Non-motile.

The species A. bovis, A. necrophorus and A. actinoides are of importance from the standpoint of veterinary medicine.

Actinomyces bovis (Discomyces bovis, Streptothrix bovis, "Ray Fungus")

There is fairly definite evidence that the "ray fungus" of actinomycosis was observed as early as 1845 by von Langenbeck[1] in material from what was evidently a case of human actinomycosis mistaken for osteosarcoma. In 1877 Bollinger[2] recognized the organism as a definite etiological factor in bovine actinomycosis and requested Harz,[3] a botanist, to make a study of it. This latter investigator accurately described the organism and gave it the name "Actinomyces."

Of the domestic animals the bovine species is most susceptible to actinomycosis. The disease is also of relatively frequent occurrence in hogs. Rarely horses, sheep and other animals are affected. Man is also susceptible to actinomycosis but, according to a number of investigators, the affection in man is caused by a species of actinomyces different from that producing the disease in lower animals.

Actinomyces bovis commonly gains entrance to the body through the agency of awns of bearded grain such as barley, wheat, oats, etc. The disease is not spread by direct or indirect contact.

Actinomycosis in cattle most frequently involves the head region, particularly the tongue and lower jaw. Thus, the disease is commonly referred

[1] von Langenbeck, Cited from Path. and Therap. of the Diseases of Domestic Animals, Hutyra and Marek, vol. I.

[2] Bollinger, Deutsch. Zeit. für Thiermed., iii, 1877.

[3] Harz, Jah. d. Muenchener Central, Thierarzneischule, 1878.

to as "wooden tongue" and "lumpy jaw." Where the tongue is affected the lesions at first appear as ulcerations, commonly located on the posterior portion of the organ. Such ulcers are ordinarily round or oval with ragged, irregular borders. In the region of the ulcers actinomycotic nodules extend into the tongue musculature. These nodules when cut through are found to contain a cheesy, gritty, greenish-yellow substance. Careful examination of this material will show the gritty condition to be due to minute, grayish, yellowish or greenish granules. Where the tongue affection has become chronic, the nodules, induration and fibrous tissue formation result in the organ becoming swollen, hard, and non-flexible, thus giving rise to the term "wooden tongue."

The affection of the maxillae ("lumpy jaw") is usually an extension of a primary infection of the tongue. Areas of the normal bone are broken down into soft, grayish, necrotic masses and at the same time a prolific, irregular new-bone formation occurs as a result of the inflammatory reaction of the periosteum. This irregular breaking down and building up of bone tissue results in a honey-comb condition of the maxillary bone, with enlargement, protrudances, or other deformity. The process may break through the skin covering the jaw, break through into the mouth, or both processes may occur. Necrotic material from such process is usually of a grayish-white or greenish-yellow color and of a semi-solid, gritty nature. The material contains the same type of actinomycotic granules as referred to in connection with the tongue lesions.

Primary actinomycosis of the lungs, udder, skin and intestinal tract are of occasional occurrence.

Morphology and Staining Characteristics. Actinomyces bovis, when present in tissue processes, forms the minute granules (0.10 to 1.50 mm. in diameter) already referred to. Microscopically these granules are found to be made up of a dense network of branching threads or filaments radiating from a central point and terminating at the periphery in characteristic, club-shaped endings. The club-like appearance of the terminal portions of the filaments is probably the result of the reaction between the organisms and the tissue of the animal host. The fine filaments usually measure about 0.40 to 0.50 micron in thickness. In some granules (older ones) numerous coccus- or spore-like bodies are observed in the net-work of filaments. These have been considered by some as products of degeneration but are possibly spores. When young these granules are rather soft and can be readily crushed between a slide and cover-glass. Granules from old lesions are frequently found calcified and consequently hard.

In making preparation for study a little "gritty" material can be mixed in

a small amount of water or physiological saline solution in a watch glass and with the aid of a hand lens the minute granules can be recognized and picked out with small forceps. These can be placed on a clean slide, crushed, fixed and stained for examination, or, better still, they may be examined unstained after simply crushing them between a slide and cover-glass. In examining unstained preparations it is best to place the granule in a drop of 10 per cent potassium or sodium hydroxide solution on a slide and then crush it with the cover glass. If the granules are calcified and hard a drop of strong acetic or hydrochloric acid can be used in place of the caustic soda or potash.

The very best way to demonstrate the rosette arrangement of the "ray fungus" is in tissue sections (see Fig. 52).

The filaments and club endings of the granules stain fairly well with the aniline dyes, carbol-fuchsin probably giving better results than some of the other stains. The filaments are uniformily Gram-positive whereas the club-shaped terminals are irregular in their reaction to such method of staining, usually appearing Gram-negative.

Cultures of Actinomyces bovis are made up of fine, branching filaments 0.40 to 0.50 micron in thickness. As in the tissue granules they stain with the ordinary aniline dyes and are Gram-positive.

Cultural Characteristics. It is ordinarily an extremely difficult task to isolate Actinomyces bovis in pure culture. Efforts to obtain pure cultures from open lesions or processes in which secondary organisms are present, are frequently futile. Occasionally a closed lesion containing only Actinomyces bovis is found and in such cases the organism can be isolated in pure culture with relative ease.

In order to obtain growth the colonies (granules) must be crushed to release the central filaments. The best procedure is to wash several granules, preferably from material obtained from a closed lesion, in sterile water or physiological salt solution in a sterile test tube. The washing should be repeated several times. After drawing off the last wash water crush the granules in the bottom of the tube with a sterile glass rod and use the crushed material for inoculation.

Actinomyces bovis is a facultative aerobe or more or less of a microaerophile. Some strains will grow in the presence of oxygen without difficulty, but a number of strains are found which thrive best under anaerobic conditions or at least in a rarefied oxygen atmosphere. The organism grows rather slowly, several days' incubation usually being required for the development of colonies of visible size. Actinomyces bovis grows well at 37.5°C. but will also develop at room temperature. Glucose added to the

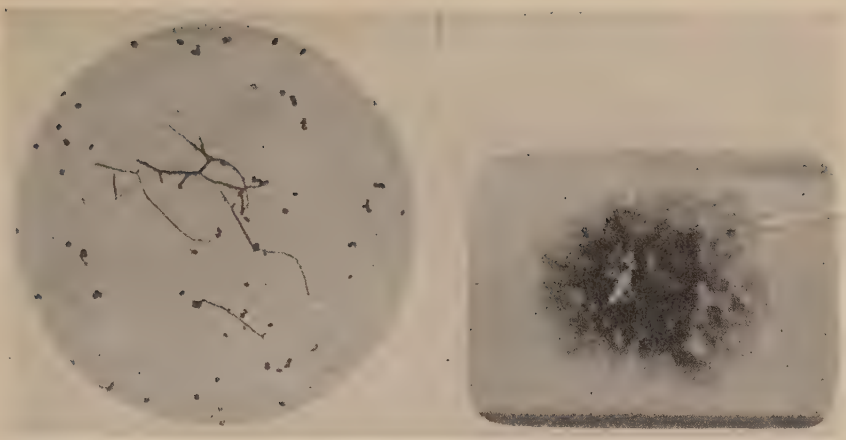


Fig. 51 Fig. 53

Fig. 51. Actinomyces bovis (Culture)
Fig. 53. Colony of Actinomyces bovis on Agar

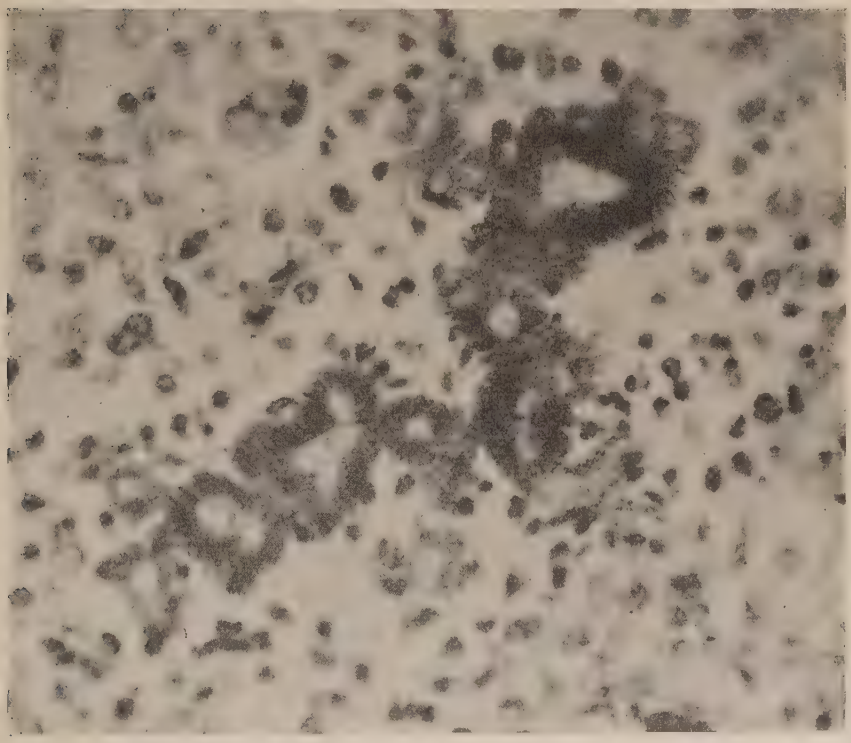


Fig. 52. Actinomyces bovis (Section of Lung of Calf)

(Photomicrograph by Maj. G. R. Callender, Army Medical Museum; Lung Section received from Dr. Fred Boerner, University of Pennsylvania.)

various media favors the growth of the organism. Blood serum is also a valuable adjunct to media for the growth of Actinomyces bovis. Culture media of slightly alkaline reaction are best suited for its propagation.

Agar. On agar, minute, raised, dew-drop-like colonies develop. These rapidly become more opaque and of a white or grayish color. Subsequently the colonies appear well raised above the surface of the medium as buds or buttons. They soon become dry or powdery and of a yellowish-red or even dark red color. The growth is firmly attached to the medium and is difficult to remove. In shake agar cultures small white colonies develop after 3 or 4 days, the growth being most abundant 5 or 10 mm. below the surface. Cultures of Actinomyces bovis give off an odor similar to that of mouldy hay.

Gelatin. Colonies on gelatin plates appear like those on agar but the medium is rapidly liquefied. In stick gelatin a yellowish growth occurs with liquefaction of the medium, the growth sinking to the bottom of the tube.

Bouillon. In bouillon, Actinomyces bovis grows in the bottom of the tube forming grayish-white or yellowish granules or flakes enmeshed in a viscous material. In some instances such granules or flakes are found in a membranous, surface growth. The bouillon is not clouded.

Litmus Milk. Actinomyces bovis propagates poorly in litmus milk. A yellowish surface growth occurs, the medium becoming coagulated and is later peptonized.

Potato. The growth of Actinomyces bovis on potato occurs in the form of small, moist, grayish colonies which subsequently become well-raised and canary yellow or reddish-yellow in color with irregular and crumpled borders and depressed centers. When the growth on potato is confluent it appears wrinkled, yellowish-gray and later reddish-yellow in color.

Nitrate Solution. Actinomyces bovis reduces nitrates to nitrites.

Loeffler's Serum Medium. This is a favorable medium for the cultivation of Actinomyces bovis. Colonies similar to those on agar develop but the color is more reddish than that noted with other media.

Dorset's Egg Medium. This is a favorable medium for the growth of Actinomyces bovis.

Resistance. Actinomyces bovis must be classed among the more resistant organisms. The organism will remain viable for several years even though kept in a dry state. A temperature of 80°C. destroys it in 10 minutes. Some authorities, however, maintain that the Actinomyces bovis will survive boiling for several minutes. Of the chemical antiseptics, bichloride of mercury is most effective, a 1:1000 solution destroying the organism

within 5 minutes. Phenol possesses only a feeble germicidal action against Actinomyces bovis.

Pathogenicity. Production of actinomycosis in laboratory animals through artificial inoculation is practically impossible. An occasional report can be found of the production of the disease in guinea pigs through artificial infection but even in those rare instances only minor, benign lesions were obtained.

Serological Reactions. No serological tests have been developed for use in connection with actinomycosis.

ACTINOMYCES ACTINOIDES (BACILLUS ACTINOIDES)

Theobald Smith,[4] in studying an outbreak of pneumonia in calves during the winter of 1917, isolated an actinomyces-like organism which he designated "Bacillus actinoides." He recognized considerable similarity between his organism and the Actinobacillus of Lignières but also pointed out that it differed in several important respects. Under the classification of the Committee of the Society of American Bacteriologists, Smith's organism is classified as a true actinomyces and designated "Actinomyces actinoides."

The disease in calves studied by Smith manifests itself as a suppurative bronchopneumonia in acute cases while in more chronic cases circumscribed portions of the lung tissue were found to have been replaced by encapsuled abscesses.

By placing a small bit of the affected lung tissue in the water of condensation in coagulated horse serum medium and then sealing the tube with sealing wax to prevent entrance of more oxygen, Smith found that after several days of incubation, minute, white flocculi developed in the water of condensation.

Actinomyces actinoides, as pointed out by Smith, differs from Actinobacillus lignièresi in that the first-named organism will not grow on ordinary culture media in unsealed tubes. Further, Actinomyces actinoides in the tissue appears as a fine bacillus whereas in culture it develops the characteristics of true actinomyces (rosettes composed of filaments and clubs). The reverse is true with Actinobacillus lignièresi, such organism showing actinomyces-like structures in tissue and not in culture. Then, Smith's organism was found producing only pulmonary lesions in calves while with Actinobacillus lignièresi lung lesions are rare and a variety of other lesions are produced in adult cattle.

[4] Smith, Theobald, Jour. Exp. Med., xxviii, 1918, p. 333.

Morphology and Staining Characteristics. In culture Actinomyces actinoides occurs as a pleomorphic organism, several forms being recognized. Preparations from the surface growth of young, special agar cultures reveal slender bacilli but after about one week the same culture is found to have developed coccoid forms. In the water of condensation of coagulated horse-serum medium the flocculi, made up of filaments and clubs, are found. These flocculi vary in size from minute particles barely within the range of visibility to flecks 1 mm. or more in diameter.

The bacillary forms stain with the ordinary aniline dyes, particularly alkaline methylene-blue and dilute carbol-fuchsin. They are Gram-negative. In young cultures, flocculi, not more than 2 or 3 days old, do not stain but the tangle of filaments of bacilli in their interior take the dye. After the flocculi become older the structure proper partially stains. The club forms can then be demonstrated but usually no filaments of bacilli are discernible.

In some of the older cultures the clubs of the flocculi are found to be surrounded by minute, highly refractile granules which, in some instances, completely obscure the club forms. Some of these granules become detached and are found free on the surface of the water of condensation.

Cultural Characteristics. As already indicated, Actinomyces actinoides is a microaerophilic organism growing only in an atmosphere of reduced oxygen tension. It grows at the regular temperature of 37.5°C. and also at room temperature.

Agar. On agar slants to which a bit of sterile guinea-pig spleen and calf serum are added (Smith), colonies varying from very minute structures to those as large as 1.5 mm. develop. They are of a straw color and coherent, it being possible to move or pick up the entire colony with the platinum loop.

Gelatin. Gelatin is not a suitable medium for the development of Actinomyces actinoides.

Bouillon. No growth occurs in broth.

Litmus Milk. No growth occurs in litmus milk.

Potato. Potato is not a suitable medium for the growth of Actinomyces actinoides.

Coagulated Serum Medium. The coagulated horse serum medium previously mentioned gives good results. The characteristic flocculi develop in the water of condensation.

Resistance. This organism is apparently no more resistant than the Actinobacillus of Lignières.

Pathogenicity. The usual laboratory animals are not ordinarily sus-

ceptible to artificial infection with Actinomyces actinoides even when inoculated with large amounts of culture.

ACTINOMYCES FARCINICUS (STREPTOTHRIX FARCINICA, NOCARDIA FARCINICA, DISCOMYCES FARCINICUS, STREPTOTHRIX FARCINI BOVIS, ACTINOMYCES NOCARDII)

In 1829 Sorillon[5] described a farcy-like disease (Farcin du boeuf) of cattle in France. This same malady, known in English as "lymphangitis farciminosa bovis," or "bovine farcy," has also been reported from India and Moritz Island. The malady is of rather rare occurrence at the present time. In France, where in the days of Sorillon's work it was of rather frequent occurrence, the disease is now seldom seen.

The etiological agent of the disease was identified in 1888 by Nocard.[6] Since its first recognition it has been given the various names mentioned above as synonyms. It has been most generally spoken of as a streptothrix but under the classification of the Committee of the Society of American Bacteriologists it becomes a member of the genus Actinomyces.

Bovine farcy is characterized by the development of a chronic and sometimes purulent inflammation of the superficial lymph glands and lymph vessels, particularly those of the inner surface of the extremities. The glands become enlarged and hard while the lymph vessels are swollen and firm, standing out like cords. Affected animals show but very little evidence of pain when the involved tissues are manipulated. Some of the lymph nodes later soften to the point of fluctuation and when incised discharge a thick, whitish, cheese-like material which has little or no odor. On the other hand a large number of the affected nodes do not soften materially but remain more or less firm. When such glands are cut through they are found to be composed largely of a tough, grayish-white, fibrous connective tissue.

The disease frequently exists for months without impairing the general health of the animal but eventually a gradual emaciation occurs which may finally result in the animal's death. Early surgical interference frequently results in the arrest of the disease but recurrences are numerous. .

Actinomyces farcinicus can be isolated from material from the affected lymph nodes and vessels.

Morphology and Staining Characteristics. Actinomyces farcinicus in the

[5] Sorillon, Cited from Kolle and Wassermann's Handb. der Path. Microorg.

[6] Nocard, Ann. de l'Instit. Pasteur, ii, 1888.

affected tissues appear as slender, branching filaments. In cultures, in addition to long filaments, shorter forms and even small, ovoid structures are noted. The organism stains well with carbol-fuchsin, gentian-violet and less satisfactorily with Loeffler's methylene-blue. A noteworthy feature is the fact that it is very definitely acid-fast. When stained according to the method of Gram the organism is decolorized, taking the counterstain.

Cultural Characteristics. Actinomyces farcinicus is an aerobe and facultative anaerobe. It grows well at the usual incubator temperature of 37.5°C. but will develop at a temperature as low as 30°C. A slightly alkaline or neutral reaction is preferable.

Agar. On agar plates, small, irregular, finely granular, opaque, yellowish-white colonies develop. These, when numerous, tend to spread out, later forming by coalescence a thick, uneven membrane. On agar slants a yellowish-white, roughened, membranous growth occurs.

Gelatin. Small, glistening, transparent colonies develop on gelatin. The medium is not liquefied.

Bouillon. In bouillon Actinomyces farcinicus gives rise to small, grayish-white granules which settle to the bottom of the tube. On the surface of the bouillon a dirty gray, dry pellicle composed of a substance which sheds water like fatty material develops. The bouillon proper is not clouded.

Litmus Milk. Actinomyces farcinicus produces no change in litmus milk.

Potato. On potato an abundant, pale yellow, dry, crumpled growth occurs.

Nitrate Solution. Nitrates are not reduced to nitrites by this organism.

Resistance. Actinomyces farcinicus is not a very resistant organism. It is destroyed in five minutes by a temperature of 80°C. A temperature of 70°C. kills it in 10 minutes. The organism is rather resistant to desiccation, remaining viable after considerable drying.

Pathogenicity. Actinomyces farcinicus is pathogenic for guinea pigs but not for rabbits. Intraperitoneal injections into guinea pigs give rise to the formation of tubercles upon the peritoneum and in some instances in the spleen, liver and kidneys. Involvement of the latter organs is more certain after intravenous injection. Subcutaneous injection results in an inflammation of the lymph glands and vessels which, in some cases, break down and suppurate.

Biological Therapy and Prophylaxis. No methods of immunization or treatment with biological products have been reported.

ACTINOMYCES NECROPHORUS (BACILLUS NECROPHORUS, STREPTOTHRIX NECROPHORA, BACILLUS DIPHTHERIAE VITULORUM, STREPTOTHRIX CUNICULI, BACILLUS FILIFORMIS)

From a veterinary standpoint Actinomyces necrophorus is one of the most important members of the group of Actinomyces. At times it has far surpassed Actinomyces bovis in importance. For example, during the world war gangrenous dermatitis among Army horses and mules, caused by Actinomyces necrophorus, ranked second in importance only to influenza. During the last six months of 1918 a total of 4036 cases of the disease occurred among army animals in the United States. Of this number 212 died or had to be destroyed on account of the disease. The weekly sick report during the period mentioned showed a ratio of over 275 animals per 1000 on sick report as a result of Actinomyces necrophorus infection.

Actinomyces necrophorus is the etiological factor of a variety of "necrobacilloses" among domestic animals. It is the cause of gangrenous dermatitis of equines, "foot-rot" and "lip-and-leg ulceration" of sheep, necrotic stomatitis of swine, calf diphtheria, dry gangrene of the udder of cows, multiple liver abscesses in hogs, cattle, sheep and horses, and a number of localized processes in different animals. It is frequently found in the intestinal tract of various animals and occasionally causes necrotic lesions in the same. In this respect the organism is found as a secondary factor in the "button ulcers" of hog cholera. As the name implies, the various conditions for which Actinomyces necrophorus is responsible are characterized by necrotic and diphtheritic inflammatory processes. Several cases of Actinomyces necrophorus infection in man are on record.

Actinomyces necrophorus was first observed by Loeffler[7] in 1884 in material from cases of calf diphtheria. Subsequently it was observed by different investigators and given a variety of names. Some considered the organism an actinomyces, others termed it a streptothrix, while still others denied that it had the characteristics of higher bacteria and classified it as a bacillus. This latter view was held by a majority of American bacteriologists until rather recently. As the matter now stands there is definite evidence of true branching by this organism so that its classification among the Actinomyces is proper.

Actinomyces necrophorus is rather widely distributed but is more prevalent in some localities than in others. It occurs in the soil in infected localities and thus frequently enters the tissues, particularly the lower extremities, through minor wounds. As a matter of fact, in the vast per-

[7] Loeffler, Mitteilungen des Kaiserlichen Gesundheitsamtes, iii, 1884.

centage of cases of Actinomyces necrophorus infections the organism enters the body through minor wounds, abrasions, etc. Gangrenous dermatitis of Army horses and mules during the world war occurred principally among animals maintained in corrals at remount depots. Rainy weather with wet, mucky corrals is conducive to such disease when the organism is present in the soil.

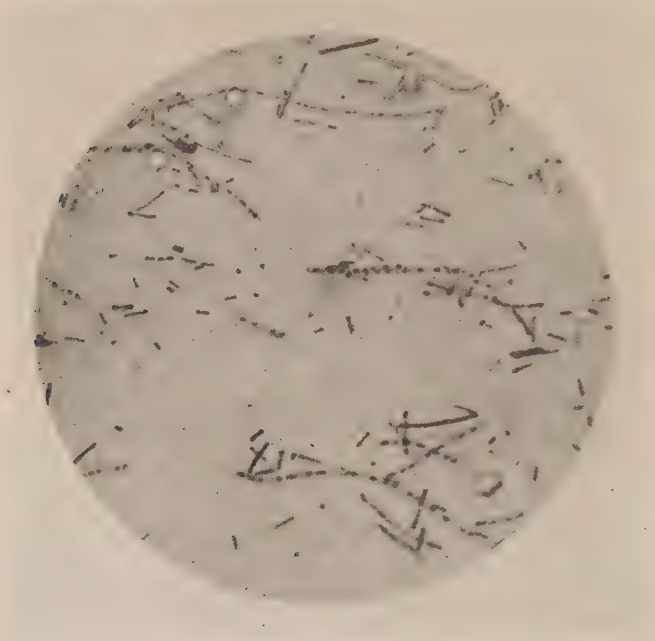


Fig. 54. Actinomyces necrophorus (Material from Case of Equine Canker)

Morphology and Staining Characteristics. Actinomyces necrophorus occurs characteristically in the form of long, beaded filaments, frequently 100 microns or more in length. It measures 0.8 to 1.5 microns in thickness. The organism, however, is distinctly pleomorphic. In addition to the long filaments shorter rods of varying size are found, some so short as to appear coccoid. The beaded appearance of the long filaments is quite characteristic and is usually sufficient to identify the organism when it is present in material from necrotic processes. In material from lesions evidence of branching is seldom found. In cultures, however, true branching can be demonstrated without difficulty. Actinomyces necrophorus is non-motile

and does not form spores. It stains with the ordinary aniline dyes and is Gram-negative. Loeffler's methylene-blue and dilute carbol-fuchsin are particularly good for bringing out its beaded appearance.

Cultural Characteristics. Actinomyces necrophorus is a strict anaerobe with an optimum temperature requirement of 34° to 36°C. It will grow satisfactorily, however, at the ordinary incubator temperature.

Isolation of this organism in pure culture is an extremely difficult task. In the first place Actinomyces necrophorus is usually present in lesions with hordes of other bacteria. This, coupled with its atmospheric requirements, make its isolation in pure culture hard. In attempts to isolate Actinomyces

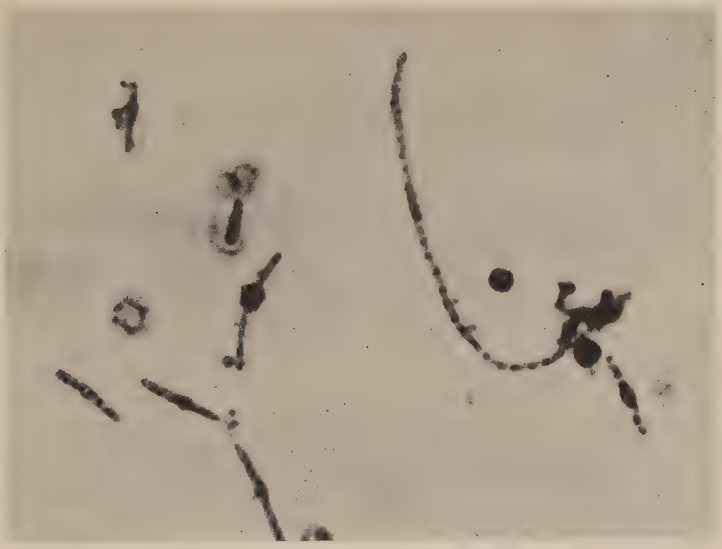


FIG. 55. ACTINOMYCES NECROPHORUS (CULTURE)

necrophorus in pure culture from material containing other bacteria, animal inoculation offers some help. For such purpose the rabbit is most suitable. The material should be inoculated subcutaneously along the median line of the abdomen. A characteristic local process develops after a few days and subsequently, in a small percentage of cases, metastatic lesions are found in the internal organs, particularly the liver. When such metastatic lesions occur Actinomyces necrophorus can usually be isolated from same in pure culture.

Of the anaerobic methods, the hydrogen replacement method is least

suited, an atmosphere of such gas not being conducive to growth. The alkaline pyrogallate method of oxygen exhaustion is satisfactory.

The organism grows best in or upon media containing blood or blood serum. Glucose also favors its growth.

Agar. On agar (serum-agar) after 48 to 72 hours, small opaque, grayish-white colonies appear. As these develop they become more opaque and appear rigid with a distinctly fimbriated border. Examination with a lens will show the colony to be made up of a felt-like mass of braided filaments, dense in the center and blending out to form a less dense, wavy, fimbriated periphery. In shake agar the colonies appear as small, fluffy structures with grayish centers, resembling small tufts of cotton.

Gelatin. Colonies on gelatin plates and in tubed gelatin resemble the growth on agar plates and in shake agar cultures respectively. The gelatin is not liquefied.

Bouillon. Bouillon is rendered uniformily cloudy and gas is produced. Cultures of the organism in bouillon and in other fluid media give off a characteristic, cheese-like odor.

Litmus Milk. Actinomyces necrophorus does not acidify or coagulate litmus milk. Cultures in this medium give off a cheese-like odor.

Potato. No growth occurs on potato medium.

Peptone Solution. Indol is formed in Dunham's peptone solution. The addition of blood serum favors growth in this medium.

Nitrate Solution. Nitrates are not reduced.

Fermentable Media. Actinomyces necrophorus does not produce gas from the carbohydrate media commonly employed.

Toxin Production. Some investigators have suggested that Actinomyces necrophorus produces a type of toxin. Such product, however, has not been satisfactorily demonstrated.

Resistance. Actinomyces necrophorus is not a very resistant organism. A temperature of 65°C. destroys it within 20 minutes. Boiling destroys it immediately. It readily succumbs to the common antiseptics in the usual strength solutions.

During the world war it was found that in cases of gangrenous dermatitis good results were obtained from acetic acid (4 per cent solution or even common vinegar) in the treatment of the affection. Investigation showed that acetic acid would destroy Actinomyces necrophorus in the dilution mentioned and that it was more effective in eliminating the organism in tissue lesions than some of the common germicidal agents.

Pathogenicity. As indicated above the rabbit is the most suitable of the laboratory animals for inoculation purposes. Mice are fairly satisfactory.

Guinea pigs are unsuited, being highly refractory to infection with this organism.

Serological Reactions. No serological tests have been developed for use in connection with Actinomyces necrophorus infections.

Biological Therapy and Prophylaxis. No biological products have been developed for use in Actinomyces necrophorus infections.

CHAPTER XXIV

Order—Actinomycetales, Family—Actinomycetaceae (Continued). Organisms of the Genus Erysipelothrix

The genus Erysipelothrix is described by the Committee of the Society of American Bacteriologists as follows:

Rod-shaped organisms with a tendency to the formation of long filaments which may show branching. The filaments may also thicken and show characteristic granules. No spores. Non-motile. Gram-positive. Do not produce acid. Microaerophilic. Usually parasitic.

At the present time there are two species in this genus. They are Erysipelothrix rhusiopathiae, the organism of swine erysipelas, and Erysipelothrix murisepticae, the causative agent of a type of mouse septicemia.

ERYSIPELOTHRIX RHUSIOPATHIAE (BACILLUS ERYSIPELATUS SUIS, BACILLUS RHUSIOPATHIAE SUIS)

In 1885 Loeffler[1] discovered the causative agent of swine erysipelas (red fever) or, as the French term it "rouget du porc," a highly important disease of continental Europe. In Germany, France, Austria-Hungary, Russia, Belgium, Holland and Denmark tremendous losses have resulted from the malady. In the United States an occasional case of the mild type of the disease has been recognized. While the existence of the disease in the United States was suspected for some few years, its occurrence was not definitely established until a few years ago when Creech of the United States Bureau of Animal Industry isolated the organism from a case of what proved to be "diamond skin disease," thus establishing beyond all doubt the existence of the disease in America.

Swine erysipelas manifests itself in three different forms. First, there is the septicemic type which is the most frequent and also the most fatal form of the disease. Then, a chronic type occurs which is characterized chiefly by an erysipelatous endocarditis with hydrothorax, pulmonary hyperemia and such other secondary conditions as usually manifest themselves in endocarditis. The third form of the disease is the urticarial type or so-called "diamond skin disease," the latter name being indicative of the

[1] Loeffler, Arbeiten aus dem Kaiserlichen Gesundheitsamte, i, 46, 1885.

shape of the skin lesions. This is the mildest form of the disease, the mortality from this type being very low.

The septicemic or acute form of swine erysipelas is characterized by sudden onset, high temperature, marked depression, vomiting and the development of characteristic hemorrhagic spots on the skin. This latter condition develops in the form of irregular, light red spots of various size. These later become a darker red in color. They are found on various parts of the body but particularly on the abdomen, in the axillary region and on the thighs. The postmortem examination of cases of erysipelas septicemia reveal only slight macroscopic changes aside from the skin lesions. There is generally a gastroenteritis, the spleen may be swollen and congested and cloudy swelling of the parenchymatous organs can, as a rule, be demonstrated. The lungs are usually edematous and hyperemic. The lymph glands of the body are swollen and hemorrhagic.

In the chronic type of the disease there develops a characteristic endocarditis marked by cauliflower-like growth with underlying ulcerations of the heart valves. In such cases, in addition to the heart lesions, the postmortem examination usually reveals hydrothorax, hyperemia of the lungs, congestion of the spleen and liver and sometimes kidney infarcts.

The urticarial type of swine erysipelas is characterized chiefly by the development on the skin of well-defined, usually rhomboidal or diamond-shaped, dark-red or purplish spots. These may be found on any part of the body but are common on the back, chest, neck and outer aspect of the thighs. Subsequently these lesions become raised and the centers may appear lighter in color. On the other hand, they may become much darker in color. In some instances an extensive gangrene of the skin develops.

In the septicemic form of swine erysipelas, Erysipelothrix rhusiopathiae can be isolated from the blood, liver, spleen, kidneys and lungs. In the chronic cases the isolation of the organism is more difficult. In such cases it can, as a rule, only be isolated from the heart lesions. Occasionally it can be recovered from the gall bladder. The demonstration of Erysipelothrix rhusiopathiae in the skin lesions of the urticarial type of swine erysipelas is frequently a very difficult procedure.

The control of swine erysipelas in countries where the malady is particularly prevalent, is rendered more difficult because of the carrier problem which exists. A considerable percentage of apparently healthy hogs "carry" Erysipelothrix rhusiopathiae in their tonsils and intestinal tracts. The gall bladder also offers a favorable habitat for the organism.

Man is susceptible to swine erysipelas when infected through tissue

injuries. Infection of man through ingestion of food or water contaminated with Erysipelothrix rhusiopathiae does not occur or at least is extremely rare. Hogs, on the other hand, are usually affected through the alimentary tract and only occasionally through tissue injuries.

There is considerable similarity between Erysipelothrix rhusiopathiae and Erysipelothrix murisepticae, the causative agent of mouse septicemia. A number of investigators have considered the two organisms identical. Such conception, however, appears to be in error, the two organisms differing in several characteristics.

Morphology and Staining Characteristics. Erysipelothrix rhusiopathiae is a small, slender rod measuring 0.20 to 0.40 micron in width and 1 to 2 microns long. The rods are, as a rule, straight, but slightly bent forms are occasionally observed. It is non-motile and does not produce spores. It occurs singly and in short chains.

The organism stains well with aqueous solutions of the ordinary aniline dyes and is Gram-positive. Occasionally, in preparations from cultures stained by Gram's method, minute granules can be demonstrated in some of the rods.

Cultural Characteristics. Erysipelothrix rhusiopathiae is a microaerophile with an optimum temperature requirement of 37.5°C. It will grow satisfactorily on most all of the ordinary culture media with the exception of potato.

Agar. On agar the growth is less luxuriant than on gelatin. Minute, dew-drop like colonies develop on agar plates incubated in an atmosphere of reduced oxygen tension. On agar slants incubated under similar conditions a scanty, grayish growth occurs.

Practically no growth occurs on the surface of culture media when incubation is carried out under the ordinary atmospheric conditions.

Gelatin. The growth of Erysipelothrix rhusiopathiae in gelatin is quite characteristic. In gelatin pour plates, hazy, bluish-gray colonies develop after about 48 hours. These colonies have a coarse, granular center which is surrounded by a zone made up of a network of branching threads, the periphery of the colony being distinctly fimbriated. In gelatin stab cultures growth starts somewhat below the surface of the medium and extends along the line of stab in the form of minute, white colonies which later become grayish or bluish-white. These subsequently fuse and from the streak horizontal branches radiate out in various directions giving the whole growth, after 8 or 10 days, a test-tube brush appearance. The gelatin is not liquefied.

Bouillon. Erysipelothrix rhusiopathiae, when cultivated in bouillon,

causes a slight turbidity which is followed by the collection of a small amount of grayish-white, flaky sediment in the botton of the tube.

Litmus Milk. Litmus milk is not a very suitable medium for the propagation of Erysipelothrix rhusiopathiae. It is unchanged by the growth of the organism.

Potato. No growth occurs on potato.

Peptone Solution. Indol is not produced in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites.

Fermentable Media. No fermentation reactions are obtained with the carbohydrate media commonly employed.

Resistance. Erysipelothrix rhusiopathiae is quite a resistant organism in several respects. While it is readily destroyed by fairly high temperatures, it is very resistant to drying, to the action of pickling fluids, and to putrefactive changes. A temperature of 72°C. destroys it in 10 minutes, 80°C. in 5 minutes. Boiling is immediately effective. It will resist drying for a month or more. Further, in a dry state it will resist direct sunlight for 10 or 12 days. In salted and pickled meats the organism will survive for 3 or 4 months. Bichloride of mercury in a dilution of 1:1000, phenol in 5 per cent solution, liquor cresolis compound in $3\frac{1}{2}$ per cent solution, and formalin in 2 per cent solution all are effective in destroying Erysipelothrix rhusiopathiae within 5 to 15 minutes.

Pathogenicity. Of the laboratory animals white mice are best suited for inoculation tests. Field mice, however, are immune. Guinea pigs, chickens, dogs and cats are also immune. The rabbit is susceptible, particularly when inoculated by the intravenous method. Subcutaneous inoculation usually results in a local process which may or may not prove fatal. The white rat is susceptible but not to the same degree as the white mouse.

Serological Reactions. A precipitin test can be obtained through the use of a suitable immune serum and antigen consisting of filtered bouillon cultures or extracts of affected tissues.

Complement-fixing bodies and agglutinins are produced.

Biological Therapy and Prophylaxis. Considerable work has been accomplished in the treatment and prevention of swine erysipelas with biological products. The results, in the main, have been quite satisfactory.

Pasteur,[2] in 1882, showed that when a culture of Erysipelothrix rhusiopathiae was passed through a number of rabbits its virulence was reduced for hogs. He developed a method of immunization which consists of the injection of 0.12 cc. of such an attenuated bouillon culture which he designated "Vaccine I." This is followed in 12 days with a like dose of a "Vaccine II" which is somewhat stronger than the first injected. Pasteur's method of immunization has been shown to be of definite value in protecting

[2] Pasteur, cited from Handb. der path. Mikroorg. Kolle and Wassermann.

against swine erysipelas. However, it is not without danger. Numerous losses, especially among the finer breeds of hogs, have, at times, resulted from its use. Even in less susceptible hogs this method of immunization often produces evidence of the mild form (urticarial) of the disease.

In 1891 Emmerich and Mastbaum[3] demonstrated that the blood serum of rabbits immunized against the swine erysipelas organism would protect against the infection. Following this report Lorenz,[4] Leclainche[5] and Schütz and Voges[6] all conducted immunization experiments in which immune serum and cultures were used. Such method (simultaneous) is the one most commonly used at the present time. Hogs are given doses of serum ranging from 3 cc. for hogs weighing 50 pounds or less up to 15 cc. for hogs of 250 pounds weight. Immediately following the inoculation of the serum an injection of virulent bouillon culture is administered in a dose of from 0.25 to 1.00 cc. according to the size of the animal. In practice some veterinarians mix the dose of serum and culture immediately before injecting it.

Good results are obtained from immune serum in the treatment of swine erysipelas when administered early in the disease. Doses of from 10 to 50 cc. repeated at intervals of 6 to 8 hours until results are obtained, are recommended.

ERYSIPELOTHRIX MURISEPTICAE (BACILLUS MURISEPTICUS, PASTEURELLA MURISEPTICA)

In 1878 Koch isolated from putrid meat an organism capable of producing a fatal septicemia in mice. The organism was termed "Bacillus murisepticus" and has since been occasionally found as the causative factor of epidemics of septicemia in mice maintained for laboratory purposes. The Committee of the Society of American Bacteriologists, in their first Manual, classified this organism as a member of the genus Pasteurella. In the second edition of the Manual it is placed in the genus Erysipelothrix. There is considerable similarity between the mouse septicemia organism and Erysipelothrix rhusiopathiae, the etiological agent of swine erysipelas. A number of investigators consider them identical. As a matter of fact they are probably not the same species but are certainly sufficiently closely related to be placed in the same genus.

As indicated, the characteristics of Erysipelothrix murisepticae are essentially the same as those described for the swine erysipelas organism.

[3] Emmerich and Mastbaum, cited from Kolle and Wassermann's Handb. der Mikroorg.
[4] Lorenz, cited from Kolle and Wassermann's Handb.der Mikroorg.
[5] Leclainche, cited from Kolle and Wassermann's Handb. der Mikroorg.
[6] Schütz and Voges, cited from Kolle and Wassermann's Handb. der Mikroorg.

CHAPTER XXV

Order—Actinomycetales (Continued), Family—Mycobacteriaceae. Organisms of the Genus Mycobacterium

The family Mycobacteriaceae is composed of four genera as follows: Mycobacterium, Corynebacterium, Fusiformis and Pfeifferella. Of these, three are of importance from a veterinary standpoint. The genus Fusiformis is of no particular concern in veterinary medicine.

The genus Mycobacterium is made up of the well-known group of acid-fast bacteria of which the organism of tuberculosis is the outstanding member. Besides Mycobacterium tuberculosis of the bovine and avian types, this genus includes another organism of considerable importance in veterinary medicine, Mycobacterium paratuberculosis, the etiological factor in Johne's disease in cattle.

The specifications for this genus, as given by the Committee of the Society of American Bacteriologists, are:

> Slender rods which are stained with difficulty, but when once stained are acid-fast. Cells sometimes show swollen, clavate or cuneate forms, and occasionally even branched forms. Growth on media slow. Aerobic.

MYCOBACTERIUM TUBERCULOSIS

Of all the microörganisms dealt with in both human and veterinary medicine there is none of greater importance than Mycobacterium tuberculosis. From the standpoint of human medicine, tuberculosis, particularly phthisis or "consumption," has from time immemorial been the "great white plague" of man. Among the lower animals, conditions which we now know were tuberculosis were also recognized many years ago but their exact nature and the analogy of such affections to "consumption" in man was not suspected in the very early days. There is evidence that pulmonary tuberculosis of cattle was observed centuries ago by Hebrew rabbis but the nature of the disease at that time was not understood. It was more or less generally believed that tuberculosis of the serous membranes ("pearl disease") of cattle was identical with syphilis in man, it being supposed that affected animals received the infection as a result of coitus to which they were subjected by syphilitic individuals.

While several investigators had earlier observed a relationship between

human and bovine tuberculosis, it was not until the end of the eighteenth century that the idea started to gain ground. In the early part of the nineteenth century a number of prominent investigators contended that pulmonary tuberculosis of cattle and man were identical. Further, several other workers expressed the conviction that "pearl disease" of the peritoneum in cattle and tubercular peritonitis in man were analogous. The subject dragged, however, until 1865. In that year a big step forward was made when Villemin[1] demonstrated that tuberculosis could be transmitted from man to animal and animal to animal by the inoculation of emulsified tubercles. This definitely proved the infectiousness of the malady and also established the relationship between the disease in man and the lower animals.

It should be noted that while Villemin's work marked the turning point of general opinion in favor of the infectious nature of tuberculosis, the contagious character of the malady had been insisted upon from time to time by various students during several centuries preceding the report of Villemin. Further, Klencke more than twenty years ahead of Villemin's work, reported the transmission of tuberculosis to a rabbit through the intravenous inoculation of an extract of tuberculous material. These contentions and findings, however, failed to receive serious consideration, the first marked change in general opinion starting with Villemin's report.

While reports confirmatory of Villemin's work were soon forthcoming from numerous investigators, the exact nature of the etiological agent was not fully established until 1882 when Robert Koch reported his epoch-making findings. Previous to Koch's work Aufrecht and Baumgarten probably saw Mycobacterium tuberculosis but they were unable to stain it satisfactorily or cultivate it. Koch fulfilled all his postulates for establishing the etiological significance of a microörganism. He demonstrated the organism in cases of tuberculosis, cultivated it in pure culture, reproduced the disease with such pure culture and again recovered the organism.

In 1898 Theobald Smith[2] published the results of his comparative study of tubercle bacilli from human and bovine sources. Smith's investigations indicated that there were definite points of difference between the organisms of human and bovine tuberculosis. In 1901 Koch[3] announced his agreement with Smith's findings. A vast amount of subsequent work by numer-

[1] Villemin, "Cause and Nature of Tuberculosis and Inoculation of Same from Man to Rabbits," Gaz. Hebdom, 1865.

[2] Smith, Theobald, Jour. Exp. Med., iii, 1898.

[3] Koch, Deut. Med. Woch., 1901.

ous investigators has left no room for doubt as regards the existence of human and bovine types of Mycobacterium tuberculosis.

Paulicki[4] in 1872 called attention to the similarity between what was later shown to be avian tuberculosis and the disease in man and cattle. Subsequently Koch demonstrated, in tuberculous lesions in birds, an organism having the same morphological appearance and staining characteristics as the causative agent of human tuberculosis. It was believed that the disease in birds was contracted through the ingestion of food or water contaminated with infectious material from affected persons or animals. A few years later, however, this view was questioned as a result of the investigations of Rivolta who failed to infect fowls through the inoculation of Mycobacterium tuberculosis of mammalian origin.

Strauss and Gamaleia, Wuertz and a number of other prominent investigators supported the contention that the causative factor of avian tuberculosis was a different type from that causing the disease in mammals. On the other hand, several other equally prominent workers failed to accept such view, believing the avian organism to be identical with the human type. At the present time there is no doubt that the avian type of Mycobacterium tuberculosis is a separate and distinct type of organism. Thus, we recognize three distinct types of Mycobacterium tuberculosis, the human, bovine and avian.

Considerable reference has, from time to time, appeared in the literature on the possibility of transmutation of types of Mycobacterium tuberculosis. While the reports are somewhat conflicting it is improbable that true transformation of type occurs.

Among domestic animals the bovine type of organism is, obviously, most important. Aside from its responsibility for tuberculosis in cattle it is the common cause of the disease in swine. Tuberculosis of sheep and goats is likewise ordinarily due to the bovine type of organism. Then, the human family is by no means immune to infection with Mycobacterium tuberculosis bovis. Infection, however, is confined largely to children, tuberculosis in adults, due to the bovine type of organism, being of rare occurrence. Among children the disease, due to the bovine organism, results from infection through the alimentary canal and commonly manifests itself as an adenitis, particularly of the cervical glands, abdominal tuberculosis or less frequently generalized tuberculosis.

The human type of Mycobacterium tuberculosis possesses but little virulence for most domestic animals. Cattle, while highly susceptible to infec-

[4] Paulicki, Magazin f. d. Gesamte Tierheilkunde, 1872.

tion with the bovine type of organism, are very refractory to the human type. While infection of hogs with the human type of organism through the ingestion of garbage contaminated by persons suffering from tuberculosis has been reported, the bovine type of Mycobacterium tuberculosis is the important factor in the disease in swine. Tuberculosis in dogs and cats is rare but when it occurs it is usually found to be due to the human type.

The avian type of Mycobacterium tuberculosis is, obviously, the important factor in tuberculosis of birds. While parrots, canaries and other small birds can be infected with the human and bovine types, chickens are practically immune to all but the avian type. Evidence, which has recently been accumulating, tends to indicate that hogs may develop tuberculosis as a result of infection with the avian type of organism more frequently than is ordinarily thought to be the case.

The typical lesion of tuberculosis is the tubercle. When young and before they start to degenerate the tubercles appear as small, grayish-white, translucent, non-vascular nodules. When present in numbers they frequently coalesce, forming larger, grayish-white or more opaque, yellowish nodules. The development of the tubercle, studied microscopically, occurs somewhat as follows: The localization of Mycobacterium tuberculosis in a particular tissue area results in the accumulation of a number of round, epithelial-like, so-called "epithelioid" cells, around the organisms. They arise chiefly from the fixed connective tissue cells as a result of the action of the products of metabolism of Mycobacterium tuberculosis. These epithelioid cells are larger than the common connective tissue cells, have vesicular nuclei and frequently are found to contain the tuberculosis organism. Mingled in with the epithelioid cells there are found large, typical giant-cells with their several nuclei arranged around the periphery of the cell. Mycobacterium tuberculosis is also frequently observed in these cells. Surrounding this area a large number of lymphocytes and an occasional polymorphonuclear leucocyte are observed. Between the cells a fibrinous matrix of coagulated exudate is present to a greater or lesser extent according to whether the process is very active or retarded in its development. After the tubercle has developed, a caseous degeneration of the central portion sets in. If the lesion heals a limiting, fibrous capsule forms and the caseated nodule becomes calcified.

MYCOBACTERIUM TUBERCULOSIS (BOVIS) (BACILLUS TUBERCULOSIS BOVIS, BACTERIUM TUBERCULOSIS BOVIS)

Tuberculosis among cattle, particularly dairy cattle, is, from an economic standpoint at least, the most important of all the infectious diseases of do-

mestic animals. The losses from this disease in the United States alone amount to millions of dollars annually. Then, as previously pointed out, young children are readily susceptible to infection with the bovine type of Mycobacterium tuberculosis so that the nation's milk supply must be carefully controlled by municipal, state and federal authorities to prevent, as far as possible, the spread of the disease by milk and other dairy products. Even with such control considerable raw milk is consumed, especially in rural districts, with the result that each year there are a considerable number of young children infected with the bovine type of Mycobacterium tuberculosis.

While it is possible for tuberculosis to be transmitted to the human family through the ingestion of meat or meat-food products, such mode of infection is rare. In the first place, meat does not ordinarily form an important part of the diet of the susceptible element of the human family—very young children. In the second place, meat is usually cooked before consumption, particularly when fed to children.

At the present time the Bureau of Animal Industry of the United States Department of Agriculture, in coöperation with the various states, is engaged in the eradication of bovine tuberculosis. This tremendous undertaking is of the utmost importance, not only from the standpoint of the livestock industry of the country but from a public health standpoint as well.

The plan of eradication involves the establishment of what are known as "Tuberculosis Free Accredited Herds." Such herds are those in which all animals of the herd have passed two successive annual or three successive semi-annual physical examinations and tuberculin tests by Federal or State veterinarians engaged in such work or by other regularly authorized and accredited veterinarians. In addition to their cattle passing such tests, herd owners agree to maintain their animals under proper sanitary conditions and to abide by the various regulations governing admission of new animals to the herd, the making of retests, keeping of records, etc. Before starting tests for accreditation the owner signs a contract in which he agrees to the slaughter of reactors with certain indemnity.

The advantage of all of this to the livestock owner lies in the fact that his herd is freed of tuberculosis and is accredited as such by the United States Department of Agriculture. This, obviously, increases the value of his herd and improves his business.

Tuberculosis of cattle is more frequently observed in mature stock than in young animals. This is not due to a greater susceptibility of older animals but to the fact that such cattle have been exposed over a longer period of time. Further, the disease is far more prevalent among dairy

cattle and animals pastured on relatively small pastures, then among cattle on the large, open ranges.

The pulmonary form of tuberculosis is the most common type of the disease in cattle. Frequently the pleura is found involved. In such cases the picture is that of "pearl disease"—tuberculosis of the serous membranes. Hard, fibrous tubercles containing caseous or calcareous material are found, sometimes in very large numbers, adherent to the thickened pleura. The pericardium is likewise frequently involved.

After tuberculosis of the thoracic organs, involvement of the abdominal structures is next in order of frequency. "Pearl disease" of the serous membranes, intestinal, liver, spleen and kidney lesions are commonly found.

In cows, tuberculosis of the udder is quite common. The disease is also occasionally found involving the genital organs. The epididymis and testicles in males, and the fallopian tubes and uterus in females, are the most common seats of infection. Ordinarily the ovaries of cows are found affected only in some of the generalized cases of tuberculosis.

The regional glands are, of course, invariably involved secondarily when the tissues they drain are affected. Tuberculosis of the lymph glands, without the involvement of other tissues, is not uncommon, being rather frequently observed in young cattle.

As previously mentioned, hogs are readily susceptible to infection with the bovine type of Mycobacterium tuberculosis. Packing house statistics reveal that large numbers of hogs coming to slaughter are infected. Considerable financial loss is sustained annually as a result of the disease. Swine become infected chiefly by way of the alimentary tract. The common practice of allowing hogs to "follow cattle" whereby they consume excrement from affected animals, and the practice of feeding them unheated, skimmed milk from cows which may be tuberculous, is largely responsible for the disease in hogs.

As would naturally be expected, the lesions in swine are found chiefly in the digestive tract and lymph glands (scrofulosis). Nodules are frequently found in the pharynx, tonsils and intestines. The retropharyngeal, cervical, mediastinal and mesenteric glands are those commonly involved. The liver and spleen are frequently affected. Miliary tuberculosis of the lungs occurs but it is by no means a common form of the disease in hogs.

Mycobacterium tuberculosis can be isolated from the various affected tissues. It should be borne in mind, however, that in some of the advanced and older processes the demonstration of the organism is frequently a very difficult matter. Young, active lesions are best suited for the demonstration of the organism.

In cattle suffering from pulmonary tuberculosis the feces frequently contain the organism. This is due to the fact that the animal will often cough up infectious material, swallow it and pass it through the digestive tract without the organism suffering injury. Thus, fecal contamination will often account for the presence of Mycobacterium tuberculosis in milk in cases where it is shown that animals are not eliminating the microbe from the udder.

In udder involvement the tuberculosis organism is frequently eliminated with the milk in enormous numbers while in other similar cases only a relatively small number are discharged.

Morphology and Staining Characteristics. Mycobacterium tuberculosis (bovis) is a pleomorphic, non-motile, non-sporulating rod measuring about 0.35 to 0.50 micron in thickness and 1.50 to 2.0 microns in length. Some forms appear broader at one end than at the other. Coccoid, elongated and branching forms are observed. From a comparative standpoint the bovine type of organism is generally shorter and not so slender as the human type. Very short rods with a few larger forms are observed. Aside from variation in size, in attempts to draw a fine distinction between the morphological appearance of the bovine type and that of the human form, a greater tendency of the latter towards irregular staining, and curvature of form has frequently been stressed. Such differences are by no means markedly distinct and constant. It is desired to emphasize the fact that definite differentiation of Mycobacterium tuberculosis of the bovine and human types on morphological grounds alone is not a simple task. Mycobacterium tuberculosis occurs singly, in pairs and in small clumps.

One of the most important characteristics of Mycobacterium tuberculosis is its staining properties. Owing to the waxy or fatty material which this organism contains, it will not stain readily with the ordinary aniline dyes. However, when once stained it retains the dye even when subjected to the action of acid-alcohol. Thus, it is spoken of as an acid-fast organism. This staining property is of great importance because ordinarily a diagnosis can be made on the strength of finding acid-fast bacteria in material of a particular nature. Of course, an absolutely unquestionable diagnosis may involve cultural work and animal inoculation tests. However, when the nature of a particular case is known and the character of the material studied is considered, the demonstration of acid-fast bacteria is generally significant. This is particularly the case when sputum or material from tissue lesions is the subject of examination. When milk and feces are examined the finding of acid-fast bacteria should be followed by guinea pig inoculation tests with a view to determining whether such organisms are Mycobacterium tuberculosis or saprophytic acid-fast bacteria.

There are several methods and modifications of methods for staining Mycobacterium tuberculosis to demonstrate its acid-fast property. Ziehl-Neelsen's method is most commonly employed. The procedure is carried out as follows: First make a thin film of the material to be examined on a slide and gently fix by heat. Cover the slide with carbol-fuchsin and heat over a flame until the preparation steams (do not boil). Continue the steaming process 3 to 5 minutes. Wash well in water. Next decolorize in acid-alcohol prepared by adding 3 cc. of hydrochloric acid to 100 cc. of 95 per cent ethyl alcohol. The decolorization is continued until no more red dye can be removed from the film. The preparation is then washed in water and counter-stained for one minute with Loeffler's methylene-blue. Mycobacterium tuberculosis will be found to have retained the red stain whereas other organisms which are not acid and alcohol fast will have been decolorized and will have taken the blue counter-stain. The carbol-fuchsin can be applied cold but in such case must be allowed to act for 15 or 20 minutes. Ordinarily it is best practice to apply heat.

Another very satisfactory method of staining Mycobacterium tuberculosis is that of Hermann's. Two stock solutions are kept on hand. One consists of a 3 per cent alcoholic solution of crystal violet, the other a 1 per cent aqueous solution of ammonium carbonate. When desired for use one part of the crystal violet solution is diluted with three parts of the carbonate solution. The preparation is flooded with this mixture and steamed gently for three minutes. Decolorize with 10 per cent nitric acid, rinse in alcohol, wash in water and counter-stain with Bismarck brown. Mycobacterium tuberculosis retains the violet dye while non-acid-fast bacteria take the counter-stain.

Gabbett recommended a method of staining acid-fast organisms in which the decolorization and counter-staining is accomplished in one operation. In this method the preparations are fixed and stained with steaming carbol-fuchsin as outlined above. They are washed and then covered with a solution prepared by adding 1 gram of methylene-blue to 100 cc. of a 25 per cent aqueous solution of sulphuric acid. This is permitted to act for one or two minutes. The preparation is then washed well in water, dried and mounted. Acid-fast bacteria retain the fuchsin while other bacteria are decolorized and take the blue stain of the acid solution. This method is not as satisfactory as the first described.

For the differentiation of Mycobacterium tuberculosis from Mycobacterium smegmatis the staining method of Pappenheim is recommended. For details of this method see section on Stains and Staining, page 91. Mycobacterium smegmatis is acid-fast and is not decolorized by aqueous solutions of acid but is decolorized by acid-alcohol.

Mycobacterium tuberculosis belongs to the Gram-positive group of bacteria.

Various methods have been advocated for the concentration of Mycobacterium tuberculosis in sputum, milk, urine, etc. With sputum antiformin can be used. This serves to break up the mucous, cells, etc. A test tube is filled about one-third full with the suspected sputum and a 10 per cent solution of antiformin added to within a short distance of the top of the tube. It is then stoppered and the mixture well shaken and allowed to stand from one-half to one hour. The mixture is then centrifugalized at high speed for 10 or 15 minutes and the supernatent fluid poured off. Sterile water is added to the tube and the same is again operated in the centrifuge for another 10 or 15 minutes. The resultant sediment is utilized for microscopic examination. As other bacteria are usually destroyed by the antiformin this method can also be utilized to advantage when cultures are made or guinea pigs inoculated.

The antiformin method is also applicable for the concentration of Mycobacterium tuberculosis in feces. With urine centrifugalization is sufficient. Milk should be centrifugalized and specimens of the upper cream zone and sediment both examined.

Cultural Characteristics. Mycobacterium tuberculosis is an aerobic organism which grows best at 37.5°C. It will, however, propagate less satisfactorily at temperatures as low as 30°C. The growth of the tuberculosis organism is exceedingly slow and initial isolations are very difficult, especially with the bovine variety. The ordinary culture media are not suitable for the primary isolation of Mycobacterium tuberculosis. Coagulated cattle or dog serum or Dorset's egg medium should be utilized. Glycerin-egg medium is not satisfactory for the initial isolation of the bovine type of organism. Material treated with antiformin or bits of tuberculous tissue removed with aseptic precautions from inoculated guinea pigs should be rubbed well over the surface of the medium. After Mycobacterium tuberculosis

FIG. 56. CULTURE OF MYCOBACTERIUM TUBERCULOSIS BOVIS ON AGAR

(bovis) has become adapted to artificial culture it will grow more readily and on media which are unsuited for its initial cultivation.

Cultures on solid media should have the stoppers paraffined or special tubes with glass tops having only a small opening should be employed. This is essential to prevent the medium from drying out before growth occurs.

As regards the reaction of media those having a pH value of 6.8 to 7.2 are satisfactory.

FIG. 57. MYCOBACTERIUM TUBERCULOSIS BOVIS

Agar. As already indicated, Mycobacterium tuberculosis (bovis) cannot be initially isolated on agar. However, after continued artificial cultivation it gradually reaches the point where it can be grown on glycerin-agar the same as the human type. On such medium, very minute colonies become discernible after about two weeks' incubation. These are at first moist and of a grayish color but soon appear dry, dull, granular and of a yellowish hue. They tend to coalesce and form a characteristic thick, wrinkled growth.

Bouillon. Glycerin broth is widely used for the cultivation of Mycobacterium tuberculosis. The amount of glycerin used in such medium by different laboratories varies from 3 to 7 per cent. For the bovine type of

organism, even after it has become well adapted to media containing glycerin, a 5 per cent content of the same should probably be the maximum employed.

In growing the organism of tuberculosis in bouillon the material inoculated must be floated on the surface of the fluid. If it sinks growth will not take place. For this reason broth is not suitable for the initial isolation of the organism. Further, it is very difficult to float even minute bits of growth from solid cultures on broth. Various methods have been suggested for transplanting growth from solid cultures to broth. Sterile bits of cork inoculated with growth from solid media and floated on the surface of the bouillon is one method advocated. Once the organism is cultivated on broth its subsequent transfer to fresh bouillon offers no particular difficulty. After getting started, the growth on broth is comparatively rapid and abundant. Transfers should be made while the culture is still actively growing and before the pellicle becomes thick.

For inoculating the broth a long, platinum loop with the loop portion bent at right angles to the rest of the wire should be used. A small piece of the young pellicle is picked up and with a straight downward motion of the loop is floated on the surface of the fresh broth.

Flasks containing broth for the growth of Mycobacterium tuberculosis should be only partially filled so that considerable surface and plenty of oxygen are available. Care should be taken not to sink the bits of pellicle when moving the flasks to the incubator. Growth usually commences within 8 to 10 days, the piece of inoculated pellicle gradually becoming larger and the growth spreading slowly over the surface of the medium. At first the forming pellicle is thin, dry and of a grayish color. Later it takes on a characteristic thickened, wrinkled appearance and becomes light yellow in color. As growth continues the pellicle becomes so heavy that part or all of it will sink if the flask is disturbed. Growing bouillon cultures of Mycobacterium tuberculosis give off a characteristic aromatic odor which is very apparent when the doors of the incubator containing a number of such cultures are opened.

Dorset's Egg Medium. This medium is particularly valuable for the isolation of Mycobacterium tuberculosis and for the maintenance of solid cultures. It has been modified by the addition of glycerin but for the isolation of the bovine type of organism the original formula should be followed. See section on Culture Media for details of preparation.

The growth of Mycobacterium tuberculosis on egg medium appears after 8 or 10 days as small, grayish, glistening colonies which later become confluent giving rise to a slightly yellowish, mealy, irregular growth.

Petroff's Medium. Petroff's medium, which consists of glycerin-beef-broth, egg and gentian-violet, is exceptionally good for the isolation of the human type of Mycobacterium tuberculosis but because of its glycerin content is not reliable for the primary cultivation of the bovine type unless the glycerin is omitted.

Blood Serum. On coagulated blood serum (dog or cattle) a slow growth similar to that seen on agar occurs.

Glycerin Potato. The human type of Mycobacterium tuberculosis grows well on glycerin potato. The bovine type will grow less luxuriantly on this medium and only after it becomes well adapted to artificial cultivation. It appears as a slight, yellowish growth, later becoming wrinkled and darker in color.

Differences Between the Bovine and Human Types of Mycobacterium Tuberculosis. The morphological differences observed between the two types of organisms have already been stated. Likewise, some of the cultural differences between the human and bovine types of Mycobacterium tuberculosis have been alluded to. First of all, the bovine type is more difficult to isolate culturally. While media containing glycerin can be used to advantage for the primary isolation of the human form, with the bovine type glycerin inhibits initial growth. Growth of the bovine organism on glycerin potato does not occur, or, at best, is very scant with recently isolated strains. This same medium is very favorable for the growth of the human type of organism even in initial culture. Another difference, first pointed out by Theobald Smith, is the change in the reaction of broth in which the two types of organisms are grown. When the bovine type of Mycobacterium tuberculosis is cultivated on slightly acid, glycerin broth the acidity of the medium is gradually reduced until it is neutral or a trifle alkaline. If growth is continued it is observed that the reaction will remain around the neutral point, fluctuating between not more than 0.1 to 0.2 per cent on either side of the "N" point. With the human type acidity of the medium is at first slightly reduced but the reaction soon starts to go the other way, the medium becoming more acid. Fluctuations occur but not to such an extent as to approximate the neutral point.

Aside from morphological and cultural variation, there is considerable difference in pathogenicity of the two types of bacteria for the lower animals. Guinea pigs are highly susceptible to both types but close observations of a number of comparative inoculation tests will reveal a tendency to a more acute course and more extensive lesions with the bovine type. The rabbit, however, is much more suitable to demonstrate this difference of virulence for laboratory animals. Intravenous injections of minute amounts of

culture are made. If the organism is the bovine type the animal will become emaciated and usually die within 5 or 6 weeks of an extensive, generalized tuberculosis in which the lungs, liver, spleen, kidneys, lymph glands and serous membranes are usually involved. If the culture is of human origin the animal will usually develop the disease in a mild form and survive a much longer period of time. If destroyed after 6 or 7 weeks lesions in the lungs and kidneys, frequently only the lungs, constitute the average findings. Another method is to inoculate a small amount of culture directly into the testicle of a male rabbit. The bovine type of organism will soon produce an extensive localized process whereas the human type will cause only a slight involvement.

Calves, of course, are readily susceptible to infection with the bovine type of Mycobacterium tuberculosis but, on the other hand, they are highly refractory to the human type.

Toxic Products. While some investigators have been inclined to believe that Mycobacterium tuberculosis produces a true toxin, the weight of evidence is opposed to such view. Reactions which may appear to be due to a true toxin have been observed, particularly in man. The author some years ago experienced a violent reaction from the accidental inhalation of a minute amount of finely powdered, precipitated tuberculin. I have since observed identical reactions in several of my laboratory assistants who have been engaged in the handling of such dry tuberculin. There was no detectable evidence whatever which would tend to indicate that any of the individuals alluded to were suffering from tuberculosis. There is every reason to believe, however, that these reactions were the result of sensitiveness gained from actual infection at some previous time with Mycobacterium tuberculosis rather than to a toxic substance in the tuberculin. Other individuals have been observed to handle this same product with utter disregard and suffer no reaction whatever. It is a well-known fact that normal guinea pigs, cattle and other animals can be given large doses of tuberculin without ill effects but when tuberculous animals are injected with such material marked reactions and even death result.

A peculiar property of Mycobacterium tuberculosis is its ability to produce lesions after it has been killed. Suspensions of killed organisms, even when washed, will produce lesions microscopically similar to those produced by the living bacteria. All of this indicates that even though Mycobacterium tuberculosis does not produce a true, soluble toxin, its cellular content and its products of metabolism are highly important.

One of the most valuable biological products the veterinary profession has at its disposal, is tuberculin. This agent has been, and still is, a highly valuable agent in the diagnosis and control of bovine tuberculosis.

Koch's early work with tuberculin was done with the hope that some such product would prove of value in the treatment and prevention of tuberculosis. Unfortunately, such hopes have not been realized. However, as a diagnostic agent, especially in tuberculosis of cattle, tuberculin is a highly valuable agent when properly used.

In the section on the Preparation of Veterinary Biological Products methods are given for the preparation of the various types of tuberculin commonly utilized by the veterinary profession. In this section it will be sufficient to merely state what tuberculin is and indicate the types employed.

Tuberculins are prepared from bouillon cultures of Mycobacterium tuberculosis grown over a period of 6 or 8 weeks. The original tuberculin of Koch is commonly known as "Old Tuberculin" or simply "O.T." It represents bouillon cultures of the organism which have been killed by heat, concentrated to one-tenth of their original volume and freed of the dead bacilli by filtration. This old tuberculin is used as the base for the preparation of various other types of tuberculins.

It is immaterial whether human or bovine strains are utilized for the production of tuberculin for human and veterinary use. While it is common practice to prepare tuberculin from cultures of both types, reactions are readily obtained with human type tuberculin in tests of animals infected with the bovine type of organism, while in man the bovine type tuberculin will elicit a reaction when infection is due to the human type of Mycobacterium tuberculosis.

The principal tuberculin tests utilized in veterinary medicine are the intradermal, subcutaneous and ophthalmic. For a long period of time the subcutaneous tuberculin test was the only official test in the United States and was utilized practically to the exclusion of other methods. During the last few years, and chiefly as a result of tuberculosis eradication activity, combination tests have come into general use. Such tests have a distinct advantage over the single tests as animals will often react to one form of test and not to another. Careful observation of the relative value of the three tests indicates that the intradermal test is the most reliable with the subcutaneous method following closely. The ophthalmic method is distinctly less reliable as a single test but it is of very definite value when used in combination with the intradermal or subcutaneous tests or both, because in certain cases of tuberculosis an ophthalmic response is the only reaction obtained.

Intradermal tuberculin consists of a dilution of Koch's old tuberculin. In applying the test to cattle a small amount (about 0.10 cc.) of the tubercu-

lin is injected intradermally, usually into one of the caudal folds at the base of the tail, the opposite fold serving as a control. This test can also be applied satisfactorily by giving the injection into the lower palpebrum. The injection of tuberculin is made with a syringe equipped with a needle of not more than $\frac{3}{8}$ to $\frac{1}{2}$ inch in length and of about 25 gauge. In positive cases reactions are usually appreciable 48 to 72 hours subsequent to the in-injection and consist of a local swelling at the point of inoculation. For full details of this and other tuberculin tests the reader is referred particularly to the publications of the United States Bureau of Animal Industry.

The subcutaneous test is based on the production of a thermal reaction in tuberculous animals when injected with tuberculin. In this test at least 3 preliminary temperatures, at not less than 2-hour intervals, are taken immediately preceding the injection of the animals, to ascertain their normal average. Adult cattle are then injected subcutaneously with a dose of tuberculin which represents 0.50 gram of Koch's old tuberculin. This amount of old tuberculin is contained in 4 cc. of the subcutaneous tuberculin manufactured by the United States Bureau of Animal Industry. The dose is the minimum and may be exceeded. Calves up to one year of age receive half the adult dose.

After the injection temperatures are taken, starting not later than the eighth hour subsequent to the inoculation. They are taken at 2-hour intervals up to 18 hours after the injection when they may be discontinued if no reaction is apparent. In positive cases the reaction consists of a rise in temperature, typical reactors showing a so-called "rainbow" temperature curve. A rise in temperature of 2°F. or more above the highest preliminary temperature, or a temperature rise above 103.8°F. showing a "rainbow" curve is indicative of a typically positive reaction.

The ophthalmic test consists of the instillation of ophthalmic tuberculin into the papebral sac. Either liquid tuberculin or ophthalmic discs can be used. In the United States discs are extensively employed. They are easy to handle and the dose is uniform. In administering the test, two applications are ordinarily made, the first instillation being in the nature of a sensitizing dose. Twelve to fourteen hours, or even several days after the administration of the sensitizing dose of tuberculin, the diagnostic dose (the same amount and the same kind of tuberculin) is instilled. Observations are commenced two hours afterwards and made at not more than 2-hour intervals over a period of at least 8 hours. A typically positive reaction is indicated by lachrymation and conjunctivitis, usually with swelling and a discharge of muco-purulent pus.

The combination test consists of combinations of all three or any two of

the tests mentioned. Aside from a combination of all three tests, the intradermic and ophthalmic tests constitute an excellent combination which is extensively employed.

In testing hogs for tuberculosis the intradermal test is the type ordinarily employed. The injection is made in the tissue of the ear.

Resistance of Mycobacterium tuberculosis. Mycobacterium tuberculosis is a rather resistant organism, especially in a dry state. While dessication will destroy some of the individual organisms in infectious material in a reasonably short time, many of them will survive for months. In putrefying material they will remain viable for weeks. In water and sewage the organism is capable of surviving for long periods of time. In ordinary milk the microbe will survive for 9 or 10 days, finally succumbing to the action of the lactic acid in the souring product. In some types of cheese it will survive for months and in butter for weeks.

Direct sunlight destroys Mycobacterium tuberculosis within a few minutes if the material containing same is spread out in a thin layer. In diffuse light they will remain alive for a week or ten days. When subjected to dry heat the organism, if in a dry state, will tolerate a temperature as high as 100°C. for 10 or 15 minutes. Exposure to such temperature for one hour definitely destroys it. In a moist state Mycobacterium tuberculosis is more readily destroyed. A temperature of 65°C. will then kill the organism within 15 minutes; 85°C. within 2 minutes. Boiling is effective in less than one minute.

In heating milk to destroy the tuberculosis organism it has been pointed out that when an open container is utilized the organisms in the upper cream layer actually receive less heat than the rest of the fluid. Thus, in heating milk in an open container the temperature should be well above the minimum required to destroy Mycobacterium tuberculosis. Approved methods of pasteurization of milk insure the destruction of this organism.

Of the chemical disinfectants corrosive sublimate, phenol, liquor cresolis compound, formalin, etc., are all effective in the usual dilutions when the organisms are present in material free from albuminous matter. When albuminous substances are present the antiseptics are much less effective. As previously indicated Mycobacterium tuberculosis is very resistant to antiformin which readily digests non-acid-fast bacteria.

Pathogenicity. It has already been stated that the guinea pig is very susceptible to tuberculosis. Such animal is highly valuable for inoculation tests. Then, the rabbit is indispensable in tests to differentiate the bovine and human types. Monkeys are very susceptible. Mice and rats are susceptible but are not very satisfactory because they rarely develop well-marked lesions.

Serological Reactions. An enormous amount of work has been done with the hope of developing a satisfactory serological test for the diagnosis of tuberculosis. The bulk of such efforts has been centered on the complement-fixation reaction. The results have been very discouraging. Complement-fixation reactions can be obtained with serum from some cases of tuberculosis but for general use the test is far too unreliable to be of much value. In tuberculosis, of the lower animals at least, the tuberculin test, properly conducted and used in conjunction with careful physical examination, constitutes the only reliable means for the diagnosis of the disease.

Biological Therapy and Prophylaxis. When tuberculin was first developed it was hoped that the product would prove of value in the prevention of tuberculosis. Then, too, it has been given rather extensive trials in the treatment of the disease but in both respects the product has been a disappointment.

In the treatment of tuberculosis there are cases on record in which some favorable results were obtained with tuberculin. On the other hand, some very detrimental results have been experienced from the use of the agent in the treatment of the disease. The dosage is an exceedingly important factor. Serious reactions and death are produced where too large doses are administered even though such doses in themselves are often very minute. Taking all the evidence into consideration, it cannot be said that tuberculin possesses any appreciable value as a curative agent.

Taking advantage of the fact that cattle are very refractory to the human type of Mycobacterium tuberculosis, Von Behring produced a "bovovaccine" from a strain of the human type of organism which had lost some of its virulence. He conducted extensive immunization tests with same. His method attracted considerable attention and was the subject of investigation by a number of students in different parts of the world. While the method produces considerable immunity, the procedure is decidedly dangerous because of the fact that vaccinated animals continue to harbor the organism in their bodies for long periods of time and frequently eliminate them with their milk, thus menacing the health of man.

MYCOBACTERIUM AVIUM (BACILLUS TUBERCULOSIS AVIUM, BACTERIUM TUBERCULOSIS AVIUM)

Tuberculosis of fowls is not an uncommon disease. As a matter of fact, it is recognized as a serious problem of the poultry industry in certain localities. Then, as has been previously indicated, attention has been recently focused on the possibility of rather frequent infection of hogs with the avian type of organism. Just how important this latter possibility is remains to be seen.

Birds are usually affected through the ingestion of food contaminated with the organism. Infection through the respiratory tract undoubtedly takes place at times but apparently this is by no means the common portal of entry. In birds the disease affects chiefly the liver and spleen. Intestinal tuberculosis and tuberculous arthritis are quite common. Strange to say, pulmonary involvement is quite infrequent in birds.

Morphology and Staining Characteristics. The avian type of Mycobacterium tuberculosis has been described by some authorities as resembling the bovine type in morphology and staining characteristics and by others as being more like the human type. From the author's observations, the avian type usually tends to resemble the human type more than the bovine. However, the line must be closely drawn to make such distinction. Mycobacterium avium is a pleomorphic organism and forms resembling both the human and the bovine organisms can be found without difficulty.

In its staining characteristics this organism does not differ materially from the mammalian types.

Cultural Characteristics. Mycobacterium avium is cultivated with a little less difficulty than the bovine and human types. It grows more rapidly than the other types and will readily propagate at temperatures as high as 42° to 44°C. The human and bovine forms will not grow at temperatures in excess of 41°C. The organism grows well on glycerin agar and potato. Unlike the human and bovine types, the avian organism will readily grow below the surface of broth, typical pellicle formation being rare.

Pathogenicity. The avian type of organism is far less virulent for guinea pigs than either the bovine or human forms. Rabbits are much more susceptible to infection with the avian type of organism than with the human form.

MYCOBACTERIUM PARATUBERCULOSIS (JOHNE'S BACILLUS, BACILLUS PARATUBERCULOSIS, BACILLUS OF ENTERITIS PARATUBERCULOSIS BOVIS)

In 1895 Johne and Frothingham[5] demonstrated an acid-fast organism in large numbers in the affected intestinal mucosa in cases of chronic enteritis or dysentery of cattle. At that time these investigators believed the disease, which had since become known by such names as "Johne's disease," "paratuberculous enteritis," "paratubercular dysentery," "chronic bacil-

[5] Johne and Frothingham, Deut. Zeitschrift für Tiermed. und vergleichende Path., xxi, 1895.

lary dysentery," etc., to be a type of intestinal tuberculosis. This view was also held by some of the later investigators. One of the first workers to differentiate between this disease and tuberculosis was Markus.[6] Subsequently Bang, Meyer, McFadyean and several others supported the contention that the disease was distinct from tuberculosis. At the present time such distinction is accepted without question.

Johne's disease occurs in Switzerland, Holland, Belgium, France, Germany, Denmark and probably other European countries. It is also of occasional occurrence in the United States.

The malady is characterized by gradual emaciation and persistent diarrhea. It runs a long course extending over several months or even a year or more, finally terminating fatally in practically all cases. Aside from evidence of emaciation, the post-mortem examination reveals no specific lesions apart from those of the intestinal tract. The intestinal mucosa, particularly that of the small intestine, is greatly thickened and presents a marked wrinkled or corrugated appearance. Mycobacterium paratuberculosis can be readily demonstrated, usually in large numbers, in material obtained by taking deep scrapings from affected portions of the intestine.

Morphology and Staining Characteristics. Morphologically Mycobacterium paratuberculosis is not very different from Mycobacterium tuberculosis. It is a more or less pleomorphic rod, the average measuring about 0.5 micron broad and from 1 to 2 microns in length. The organism is nonmotile and non-sporulating. The rods are found singly, in pairs, and in material from lesions they are very frequently found in groups or clumps. They are acid-alcohol fast and frequently stain irregularly. The organism cannot be satisfactorily stained in the ordinary way with the usual aniline dyes. It is Gram-positive. In staining this organism the technic described for Mycobacterium tuberculosis is applicable.

Cultural Characteristics. Mycobacterium paratuberculosis is a very difficult organism to cultivate artificially. While first observed in 1895, it was not grown on artificial culture media until 1912. In that year Twort and Ingram[7] succeeded in cultivating it on a glycerin-egg medium to which had been added 0.50 to 1.00 per cent killed Mycobacterium tuberculosis of the human type. Such type of medium had previously been found of value in the cultivation of the leprosy organism.

It has been subsequently found that extracts of Mycobacterium tuberculosis or even the broth from bouillon cultures of same, when incorporated in

[6] Markus, Zeitschrift für Tiermed., viii, 1904.

[7] Twort and Ingram, Proc. Royal Soc., lxxxiv, 1912.

egg medium, agar, coagulated blood serum or liver bouillon, render such media suitable for the cultivation of Mycobacterium paratuberculosis.

Hastings, Beach and Hadley[8] report good results with a medium prepared as follows: To filtered broth cultures of the human type of Mycobacterium tuberculosis, sterilized by heating for two hours in the Arnold sterilizer, add 15 grams of agar, 2 grams of beef extract, 5 grams of peptone, 2.5 grams of potassium acid phosphate and 24 cc. of glycerin, per liter of filtered culture. Adjust the reaction to plus 1.5 to phenolphthalein, tube and sterilize.

After once obtained in artificial culture, subsequent generations of the organism grow more readily and more abundantly. It can then be cultivated in glycerin bouillon without the addition of preparations of Mycobacterium tuberculosis.

The optimum temperature for the organism of Johne's disease is a little above the ordinary incubation temperature—about 39°C. It will, however, propagate at temperatures as low as 27°C. and as high as 43°C.

Egg Medium. When inoculated on egg medium and incubated for several days, very minute, slow-growing, dry, grayish-white colonies appear. Later, as these colonies become older and larger in size, they take on a wrinkled appearance.

Bouillon. In broth Mycobacterium paratuberculosis grows in clumps which sediment to the bottom of the tube leaving the fluid above clear. Cultures which have become adapted to artificial cultivation usually produce a wrinkled, membranous pellicle.

Resistance. Mycobacterium paratuberculosis is similar to the organism of tuberculosis in its resistance to the elements, heat and chemical germicides.

Pathogenicity. None of the smaller animals commonly employed for laboratory inoculation tests are susceptible to infection with Mycobacterium paratuberculosis. This fact serves to distinguish the organism from that of tuberculosis.

Biproducts. This organism does not produce a true toxin. However, a preparation known as "Johnin," can be made from bouillon cultures in essentially the same way as tuberculin and is of value as a diagnostic agent. In attempting to prepare such product it is best to employ a culture which can be grown in plain glycerin broth as, obviously, "Johnin" prepared from bouillon cultures containing products of the tuberculosis organism, will provoke reactions in tuberculous animals and subsequently necessitate a tuberculin test for differential purposes.

[8] Hastings, Beach and Hadley, Jour. A. V. M. A., no. 4, January, 1918.

It has been shown that a tuberculin made from cultures of the *avian* type of Mycobacterium tuberculosis will elicit a reaction in non-tuberculous animals affected with Johne's disease. Where such tuberculin is employed, positive reactions must be checked for specificity for Johne's disease by a subsequent test for tuberculosis, using tuberculin prepared from the human or bovine type of Mycobacterium tuberculosis. In the presence of tuberculosis, the use of tuberculin for the diagnosis of Johne's disease is, obviously, out of the question.

Serological Reactions. Complement-fixation and agglutination reactions have been obtained in cases of Johne's disease and with the serum of laboratory animals given injections of cultures of Mycobacterium paratuberculosis. Results from such tests are irregular and far from satisfactory. Further, it has been found that cross fixation and agglutination reactions are obtained with antigens prepared from other acid-fast bacteria.

OTHER ACID-FAST BACTERIA

There are a number of acid-fast bacteria which occur as saprophytes. Of these the commonest known are *Mycobacterium smegmatis*, *Mycobacterium butyricum* and *Mycobacterium phlei.*

Mycobacterium smegmatis (Bacillus smegmatis) is found about the genitals of man and some of the lower animals. As it is non-pathogenic it is only of importance in that it might be present in urine or other material examined for Mycobacterium tuberculosis and may be mistaken for such organism. It is acid-fast but is decolorized by the method of Pappenheim (page 91) in which alcohol and rosolic acid are employed. Further, guinea pig inoculation tests will serve to eliminate it as an organism of tuberculosis.

Mycobacterium butyricum (butter bacillus) has been isolated from butter and milk. Most strains of this organism are not near so tenacious in their acid-fast property as the tuberculosis organism. It grows on a greater variety of culture media. Further, growth of this organism on the types of media most favorable for Mycobacterium tuberculosis is far more rapid and luxuriant than the latter. Mycobacterium butyricum is slightly pathogenic for guinea pigs. However, the affection of such animals is of such a benign character as to offer little difficulty in differentiating it from real tuberculosis.

Mycobacterium phlei (timothy hay bacillus) is an organism occasionally found in hay and sweepings from hay-lofts. Thus, it sometimes finds its way into milk. The organism is non-pathogenic and is rather easily recognized by its rapid and abundant growth on most of the ordinary culture

media. On agar, a luxuriant, reddish or reddish-yellow growth occurs within the course of several days.

Aside from the acid-fast bacteria already referred to, there is one other important member of the group—*Mycobacterium leprae*, the causative agent of leprosy of man. This organism is not of importance in veterinary medicine but is merely mentioned because it, with the three types of Mycobacterium tuberculosis and the organism of Johne's disease, constitute the important members of the acid-fast group.

CHAPTER XXVI

Order—Actinomycetales, Family—Mycobacteriaceae (Continued)
Organisms of the Genus Corynebacterium

The specifications for this genus, as given by the Committee of the Society of American Bacteriologists, are:

> Slender, often slightly curved, rods with a tendency to club and pointed forms, with branching forms in old cultures. Barred, uneven staining. Not acid-fast. Gram-positive. Non-motile. Aerobic. No endospores. Some pathogenic species produce a powerful exotoxin. Characteristic snapping motion is exhibited when cells divide.

The important member of this genus is the type species, Corynebacterium diphtheriae, the causative agent of human diphtheria. This species is not considered in detail in this work because, from a veterinary standpoint, it is of but little interest. It should be noted, however, that lower animals, particularly the cat, have at times been proved to be carriers of the organism of human diphtheria. While noting this fact, it is also desired to emphasize the importance of proving, by immunological tests with guinea pigs, the identity of what appear to be diphtheria organisms isolated from the lower animals. Cases of carriers of the real diphtheria organism among lower animals have, as stated, been definitely proved but, on the other hand, diphtheroid bacteria have undoubtedly been mistaken for Corynebacterium diphtheriae in a number of instances.

The only species in this genus of appreciable importance in veterinary medicine is Corynebacterium pseudotuberculosis (Preisz-Nocard bacillus). This organism is classified by the Committee of the Society of American Bacteriologists as "Corynebacterium ovis." As it is rather well established that this organism is not only responsible for the so-called "pseudotuberculosis" or caseous lymphadenitis of sheep, but is also the etiological factor in ulcerative lymphangitis of horses, the specific name "ovis" is a misnomer. As the organism has been commonly known as "Bacillus of pseudotuberculosis," in this text it has been designated "Corynebacterium pseudotuberculosis."

CORYNEBACTERIUM PSEUDOTUBERCULOSIS (PREISZ-NOCARD BACILLUS, BACILLUS OF PSEUDOTUBERCULOSIS)

In 1891 Preisz and Guinard[1] described a tuberculosis-like disease in sheep, designating the malady "pseudo-tuberculosis." From a kidney lesion in one of the sheep Preisz isolated the organism now under consideration and demonstrated it to be the causative factor of the disease.

Pseudotuberculosis or caseous-lymphadenitis has been reported from Germany, France, Australia, New Zealand, United States, Argentina, Chile and undoubtedly has occurred in various other countries. In the United States the disease was the subject of careful study by Nörgaard and Mohler.[2]

Pseudotuberculosis of sheep is characterized chiefly by an inflammatory enlargement of the lymph glands, followed by caseous degeneration. The glands most commonly found involved are the prescapular, precrual, inguinal, bronchial, mediastinal and sublumbar. In addition to the glands, caseous foci are frequently found in the lungs, spleen, liver and kidneys. Calcified lesions are rarely observed. Cross section of the lesions frequently shows the nodule to be laminated. In addition to sheep, the disease is observed in goats.

The year following Preisz and Guinard's report (1892) on pseudotuberculosis of sheep, Nocard[3] described an ulcerative lymphangitis of horses which had been mistaken for the farcy form of glanders. From these cases he isolated the same organism as that found in pseudotuberculosis of sheep. The relationship between the two organisms, however, was not recognized until later years.

Ulcerative lymphangitis of horses has been reported principally from France although it undoubtedly occurs in various other countries. So far as known, it is not prevalent in the United States. In this disease the subcutaneous lymphatic system is involved. A pronounced inflammation of the lymph vessels with enlargement and later ulceration of the lymph nodes occur. The process commonly affects the extremities, particularly the hind legs, but in advanced cases lesions may be found on the trunk, head and neck and in deep-seated glands. The disease runs a persistent, chronic course.

Morphology and Staining Characteristics. This organism is a rather short, slender, non-motile, non-sporulating rod. In size the organism is about 0.5 micron broad and varies from 1 to 2.5 or 3.0 microns in length. It is

[1] Preisz and Guinard, Jour. de méd. vét. et de Zootech., Lyons, 1891.
[2] Nörgaard and Mohler, Rpt. U. S. Bureau of Animal Industry, 1899.
[3] Nocard, Bull. de l'Instit. Pasteur, 1893, 116.

quite pleomorphic, club-shaped and plump, ovoid forms being frequently observed. Corynebacterium pseudotuberculosis occurs singly, in pairs, and frequently in groups. It can be stained satisfactorily with the ordinary aniline dyes and is Gram-positive. In stained preparations it will be noted that some forms appear striated, resembling, in such respect, the organism of human diphtheria.

Cultural Characteristics. Corynebacterium pseudotuberculosis is an aerobe and facultative anaerobe. Its optimum temperature requirement is about that of the body, 37.5°C. Practically no growth occurs at temperatures appreciably lower. It will develop in or upon media of neutral, slightly acid or slightly alkaline reaction. The growth of primary culture is slow but after becoming adapted to artificial media it develops more luxuriantly. Media containing glycerin are not as favorable as media without same. Blood serum is a valuable adjunct to media for the propagation of this organism.

Agar. On agar plates, small, gray or grayish-white, scale-like colonies develop within 24 to 36 hours. After several days these become larger and appear dull and granular. The centers of such colonies are depressed and the borders serrated. Further, the surface of the colony shows a series of concentric rings between the center and periphery. Slant agar cultures reveal a thin, opaque, grayish-white, confluent growth which is at first somewhat moist, later becoming dry.

Gelatin. Gelatin is not a favorable medium for the growth of Corynebacterium pseudotuberculosis because it requires for satisfactory development a temperature approximating that of the body. No liquefaction of the gelatin occurs in instances where one succeeds in obtaining slight growth in this medium.

Bouillon. Bouillon becomes turbid shortly after inoculation but soon clears. A granular sediment collects in the bottom of the tube while on the surface of the medium a dry, rigid, grayish-white pellicle forms.

Potato. This is not a satisfactory medium for the propagation of the organism here considered.

Litmus Milk. Litmus milk is unchanged by Corynebacterium pseudotuberculosis.

Peptone Solution. This organism does not produce indol in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites by Corynebacterium pseudotuberculosis.

Loeffler's Serum Medium. This is a very satisfactory medium for the cultivation of the Preisz-Nocard organism. The colonies are small, serrated and of a yellowish-color.

Fermentable Media. Acid is produced from dextrose.

Toxin. Bouillon cultures and culture filtrates have been demonstrated to be toxic for sheep, guinea pigs and rabbits.

It was reported, that as the serum of horses suffering from ulcerative lymphangitis contained substances capable of neutralizing the toxin of the Preisz-Nocard organism, diagnosis of such disease could be made by immunological tests carried out on guinea pigs. Such tests were used to some extent for the diagnosis of equine ulcerative lymphangitis during the world war. It has since been determined that the serum of a large percentage of normal horses, with no history whatever of ulcerative lymphangitis, will neutralize the toxic substance in cultures of Corynebacterium pseudotuberculosis. The test is, therefore, obviously unreliable.

Resistance. Corynebacterium pseudotuberculosis is not a very resistant organism. It is destroyed within 15 minutes by a temperature of 65°C. Boiling kills it immediately. The common disinfectants are effective in the usual strength solutions.

Pathogenicity. Guinea pigs frequently develop an orchitis when inoculated intraperitoneally with Corynebacterium pseudotuberculosis and die within a week or ten days. Rabbits, injected intravenously, become greatly emaciated and die after several weeks. In such animals caseous foci can be demonstrated in the liver, lungs, lymph glands, etc. The organism is also pathogenic for mice.

Biological Therapy and Prophylaxis. Vaccines, bacterins, etc., have been utilized in some instances in connection with the control of the maladies produced by Corynebacterium pseudotuberculosis. The results reported have been variable.

CHAPTER XXVII

Order—Actinomycetales, Family—Mycobacteriaceae (Continued). Genus Pfeifferella. Pfeifferella Mallei

As defined by the Committee of the Society of American Bacteriologists this genus includes:

> Non-motile rods, slender, Gram-negative, staining poorly, sometimes forming threads and showing a tendency toward branching. Gelatin may be slowly liquefied. Do not ferment carbohydrates. Growth on potato characteristically honey-like.

This genus is made up at present of but one species, the well-known organism of glanders, Pfeifferella mallei.

PFEIFFERELLA MALLEI (BACILLUS MALLEI, BACTERIUM MALLEI)

The history of glanders is quite interesting. It is one of the oldest known diseases, its recognition dating back several centuries B.C. In spite of the fact that the malady had been looked upon as contagious for hundreds of years, several prominent French investigators during the middle of the nineteenth century came out with the statement that the disease was not infectious. Although this contention was contested by other authorities, regulations and measures for the control of glanders in France were permitted to lapse. The result of this, obviously, was a tremendous increase in the spread of the disease. After tremendous losses had occurred it became apparent that a mistake had been made and measures were again inaugurated for glanders control.

Babes, in 1881, was probably the first to observe the glanders organism in material from lesions. The following year (1882) Loeffler,[1] assisted by Schütz, isolated the causative factor.

During the days when the horse was the chief factor in transportation, glanders was very frequently a serious problem, particularly in the large cities. At the present time the malady is very much less of a problem. This is due to several factors. In the first place, there are fewer horses on our city streets, hence, chance of exposure to the infection is less. Then, the common watering trough, a potent factor in the spread of glanders, has been eliminated by a great many cities and horses on the streets are watered

[1] Loeffler and Schütz, Deutsche medizinische Wochenschrift, Berlin, 1882.

from buckets carried by the driver. Further, the average standard of sanitary requirements for the maintenance of stables has been considerably raised. Then, competent veterinary service is undoubtedly employed to a greater extent and improved methods for the early diagnosis of the disease are made use of at regular intervals. Municipal, State and Federal activity in the control of animal diseases has also contributed largely to the decrease in the prevalence of the disease.

While all of the above is true, it should be borne in mind that glanders is by no means eradicated. Its continued suppression depends on the early detection and prompt and proper disposal of "first" cases. Where large groups of animals are congregated the veterinarian must be constantly on the alert to prevent the introduction of the disease by outside or newly introduced animals. Further, aside from newly introduced animals, horses already on the premises should be subjected to the mallein test at fairly frequent intervals to insure the early detection of the disease in case the infection gets in indirectly.

Glanders is ordinarily a chronic disease of the equine species, in which characteristic nodules or tubercles develop. These later undergo a cheesy degeneration and, if located in the cutaneous tissue, ulcerate. Three forms of the disease are recognized—pulmonary, nasal and the cutaneous or farcy form.

Frequently in the pulmonary form no appreciable external evidence of the disease is manifested in the early stages. The animal will sometimes go for months without showing signs of disturbed health. Finally, however, its general condition becomes poor and there is evidence of pulmonary impairment. On the other hand, in some cases the animal in the early stages of the disease shows definite symptoms of a lobular or bronchopneumonia. Postmortem examination in cases of pulmonary glanders reveals small nodules or tubercles and frequently areas of consolidation of variable size. In the beginning these areas are of a reddish-brown color. Later they commence to undergo degeneration and then they show a yellowish, cheesy center surrounded by a firm, reddened zone of inflammation which has a characteristic yellowish, gelatinous-like sheen. In cases where the involvement is extensive, some of the consolidated areas become confluent and form firm, sarcoma-like "glanderous growths." On cut surface they appear either homogeneous and of a grayish-red or white color, or they appear honeycombed, the cavities containing a semifluid, cheesy or purulent material.

In nasal glanders the disease may start with a slight discharge which might be mistaken for a simple rhinitis. Subsequently the discharge be-

comes more copious and purulent. It may be bilateral but is more often unilateral. Small, grayish-white or yellowish nodules develop in the lower portion of the nasal cavity, especially on the Schneiderian membrane. These latter break down forming ulcers which show raised, irregular borders. If the ulcers heal there is usually left a rather characteristic, star-shaped scar which is at first pink, later becoming white. The submaxillary glands are invariably involved in nasal glanders.

FIG. 58. PFEIFFERELLA MALLEI

In cutaneous glanders or farcy, nodules are found in the skin and subcutaneous tissues. The lymph spaces between affected nodes stand out as thickened cords. The nodes break down and rupture and after discharging their yellowish-gray pus develop into rather deep, crater-like ulcers. These bleed easily and are very slow to heal.

Irrespective of the form glanders assumes, postmortem examination, in practically all cases, reveals lesions in the lungs. Lesions in the spleen and liver are also of rather frequent occurrence. Regional lymph glands of affected organs or tissues are, obviously, usually involved.

Pfeifferella mallei occurs in the nasal discharge and in affected tissues. It is frequently difficult, however, to demonstrate the organism in some lesions, especially the older ones, either microscopically or culturally.

Man is readily susceptible to glanders, the disease usually running a fatal course.

Morphology and Staining Characteristics. Pfeifferella mallei is a slender, non-motile, non-sporulating rod with rounded ends. It varies in size from 0.31 to 0.50 micron in thickness and from 1.5 to 5 microns in length. Some slightly curved forms are seen. Occasionally short, plump forms are noted. In the tissues the organism is usually found singly, in pairs and in groups. In culture preparations they are found singly, in pairs and occasionally in branching filaments. The organism stains but poorly with the aqueous solutions of aniline dyes. Carbol-fuchsin and Loeffler's alkaline methylene-blue give rather satisfactory results. The microbe frequently shows irregular staining, some of the rods appearing to possess deep-staining granules. It is Gram-negative.

Cultural Characteristics. Pfeifferella mallei is an aerobe and facultative anaerobe. However, under anaerobic conditions its growth is very poor. Its optimum temperature requirement is 37.5°C. At temperatures below 20°C. and above 44°C. it will not propagate. Culture media of slightly acid reaction are best suited for its growth. It will, however, grow satisfactorily in or upon media of slightly alkaline or neutral reaction. The addition of glycerin to culture medium enhances its value for the cultivation of the glanders organism. Blood serum likewise aids growth. Potato is an excellent medium.

Agar. On agar plates, moist, grayish-white, translucent, ropy colonies with regular borders appear within 24 hours. These later become yellowish or brownish-yellow in color. On agar slants a glistening, moist, ropy, grayish-white growth develops which later becomes slightly yellowish in color.

Gelatin. Gelatin is not very well suited for the growth of Pfeifferella mallei. Colonies on this medium are smaller than on agar. The medium is not liquefied.

Bouillon. Pfeifferella mallei slowly clouds bouillon. Subsequently a slimy or ropy sediment forms and, if undisturbed, a thin pellicle is produced. The turbidity persists.

Litmus Milk. Irregular results are frequently obtained with Pfeifferella mallei when grown in this medium. Ordinarily coagulation occurs after a week or more. In some cases the litmus is reduced, in others it is not.

Potato. The growth of the glanders' organism on potato medium is quite characteristic. After 36 to 48 hours, pale yellow, honey-drop-like colonies are apparent. As the growth ages it becomes more abundant and gradually becomes darker assuming a reddish-yellow or chocolate color.

Around the growth the medium sometimes has a faint greenish tinge. The pigment changes are best noted when the potato medium is not too acid. In preparing the medium it is advisable to soak the potato plugs for one-half to one hour in a 0.50 per cent sodium bicarbonate solution immediately before placing them in the tubes for sterilization.

Peptone Solution. This organism does not produce indol in Dunham's peptone solution.

Nitrate Solution. Nitrates are not reduced to nitrites.

Loeffler's Serum Medium. Pfeifferella mallei grows well on coagulated serum medium. Moist, viscid, yellowish colonies develop after 36 to 48 hours.

Resistance. Pfeifferella mallei is not very resistant. A temperature of 55°C. destroys it within ten minutes. It is killed by a temperature of 75°C. in five minutes. Direct sunlight will destroy the organism in a few hours. Pfeifferella mallei usually does not resist drying for more than 2 or 3 weeks but in some instances it may survive 2 or 3 months. It will remain alive in 25 to 30 per cent glycerin for about two weeks without loss of virulence. Glycerin, therefore, is a suitable preservative in which to ship suspected glanders' specimens to the laboratory for bacteriological examination. The common disinfectants, in usual strength solutions, are effective in the destruction of this organism within 10 or 15 minutes.

Pathogenicity. Guinea pigs, rabbits, dogs and cats are susceptible. The guinea pig, however, is by far the best animal for inoculation purposes. The so-called "Strauss test" for the diagnosis of glanders, consists of the intraperitoneal injection of a male guinea pig with a small amount of suspected material. If Pfeifferella mallei is present an orchitis starts to develop within 3 or 4 days and in an additional few days the testicles will abscess and break. Before they rupture it is best to destroy the animal and make cultures from the testicle opened with aseptic precautions. As a matter of fact, the animal can be chloroformed and cultures made just as soon as the orchitis is well marked. In addition to the testicular involvement, inoculated guinea pigs show lesions in the liver, spleen, pancreas, lungs, etc.

Serological Tests. Highly satisfactory results in the diagnosis of glanders have been obtained with the agglutination and complement-fixation tests. These tests are extensively used in glanders control work, both alone and supplementary to mallein tests. In the latter cases the serological tests are used to clear up indefinite mallein reactions. The complement-fixation test for glanders is probably the most satisfactory of all complement-fixation tests. The precipitin and conglutination tests have been utilized for

the diagnosis of glanders but are not near so satisfactory as the agglutination and complement-fixation tests.

The methods of conducting the serological tests for glanders are given in the section on Serology.

Mallein Tests. The mallein test for glanders is an allergic reaction similar to the tuberculin test. Preparation of mallein was first accomplished by Helman and Kalning.[2] These investigators did not employ the method now used but prepared their product from potato cultures of Pfeifferella mallei.

In the United States for a rather long period of time the subcutaneous mallein test was used practically to the exclusion of other forms of such tests. Then the ophthalmic test came into favor and was recognized by the Federal Bureau of Animal Industry as an official test. With the world war the intradermal (intrapalpebral) test was introduced as the official test for Army animals and subsequently has been utilized on a large scale by the Army Veterinary Corps and civilian veterinarians.

The extensive use of mallein for the diagnosis of glanders has proved, beyond all doubt, that such product is a highly valuable agent and a tremendous factor in the control and eradication of the disease.

Biological Therapy and Prophylaxis. The use of mallein in the treatment of horses affected with glanders has been reported by various investigators. While some favorable reports have been made the great bulk of evidence indicates that glanders cannot be satisfactorily treated or prevented with mallein or any other known agent.

[2] Helman and Kalning, cited from Deut. tierarztliche Wochenschrift, 65, 1910.

CHAPTER XXVIII

Order—Spirochaetales, Family—Spirochaetaceae. Organisms of the Genus Borrelia

Under the order Spirochaetales there is one family—Spirochaetaceae. This family is, at present, composed of six genera as follows: Spirochaeta, Saprospira, Cristispira, Borrelia, Treponema and Leptospira.

In so far as veterinary medicine is concerned the entire order of Spirochaetales is, at the present time, of but little importance. A number of species of lower animals harbor a variety of microörganisms of this group but there are but few specific diseases of domestic animals at present known to be due to such types of microbes. The rat and mouse are carriers of several of the organisms of this order pathogenic for man.

The genus Borrelia contains several members, which, from a veterinary standpoint, are worthy of mention. The specifications for this genus, as given by the Committee of the Society of American Bacteriologists, are as follows:

> Small, parasitic, spiral forms; flexible, with terminal filaments. The spirals are large, wavy, three to five in number.

Three species of this genus will be briefly referred to, namely, Borrelia gallinarum, Borrelia theileri and Borrelia hyos.

In examining material for the presence of organisms of this group the dark field method is widely used and usually gives considerably more information as to morphology, movement, etc., than can be obtained from a study of stained preparations.

In fixed preparations the silver impregnation method of Levaditi is a very satisfactory method of demonstrating these organisms. The India ink and Fontana's methods are also very valuable.

Borrelia gallinarum (Spirochaeta anserina, Spirochaeta gallinarum)

In 1891 Sakharoff[1] reported the finding of a spirochaete as the causative factor of a septicemic disease of geese in Russia. He designated the organ-

[1] Sakharoff, Annales de l'Institut Pasteur, v, 1891, 564.

ism "Spirochaeta anserina." In 1903 Marchoux and Salimbeni[2] reported the isolation of a similar organism in an outbreak of disease among fowls in Brazil and named the causative factor "Spirochaeta gallinarum." For a time it was thought that the two organisms were different species but subsequent investigations have demonstrated that they are identical.

Borrelia gallinarum is responsible for spirillosis or spirochactosis of geese, chickens and ducks. The disease manifests itself as an acute septicemia. Affected birds develop fever, marked weakness, drowsiness, diarrhea, emaciation and, in some cases, paralysis of the extremities. The disease is fatal in a high percentage of cases. Postmortem examination reveals an enlarged spleen, swollen liver with evidence of fatty degeneration and a fibrinous epicarditis. Borrelia gallinarum can be demonstrated in the blood during the course of the disease but it disappears shortly before death. The infection is transmitted by ticks, particularly Argas miniatus and Argas persicus.

Morphology and Staining Characteristics. Borrelia gallinarum is a long, spiral organism measuring from 8 to 20 microns in length. It is not closely spiralled, there being a distance of from 1 to 1.4 microns between the coils. Terminal granules are frequently observed and in cultures a week or more old, small, coccoid bodies are found. The organism is actively motile. Borrelia gallinarum is more easily stained than some other members of this group. Carbol-fuchsin, gentian-violet, Giemsa stain, etc., give satisfactory results. The silver impregnation method of Levaditi and the India ink method give the most excellent results.

Cultivation. This organism can be cultivated but not without some difficulty. The most satisfactory results are obtained with acitic fluid to which bits of sterile kidney from a rabbit, or chicken muscle are added. Such media should be layered with paraffin oil. A temperature of 37.5°C. is apparently the optimum. In culture the Borrelia form develops rather rapidly during the first week then various degenerative or involution forms, particularly coccoid bodies, are observed. Cultures containing practically nothing but such degenerative or involution forms are still capable of producing the disease, the cultures retaining their virulence for 4 or 5 weeks.

Pathogenicity. The rabbit, guinea pig, white rat, etc., are not suitable for inoculation tests. Various birds, however, are readily susceptible. Chickens, ducks, geese, canaries, sparrows, etc., can be utilized. Young chickens are more satisfactory than adult birds, especially when the material for inoculation is from a different species.

Serological Reactions. Borrelia gallinarum infection stimulates the pro-

[2] Marchoux and Salimbeni, Annales de l'Institut Pasteur, xvii, 1903, 569.

duction of agglutinins which will agglutinate the organism in young cultures (48 to 72 hours old). The agglutination test, however, has not come into practical use in Borrelia gallinarum infections.

Biological Therapy and Prophylaxis. Affected birds have been treated with immune serum with satisfactory results. Horses can be employed for the production of such agent. This serum confers a passive immunity which will protect susceptible birds from infection for a short period of time.

Marchoux and Salimbeni have shown that fresh blood from affected birds can be rendered safe for inoculation into susceptible birds by heating it at 55°C. for five minutes and that the injection of such material will render the inoculated birds immune. The heating process must not extend beyond the 5-minute period. Ten minutes exposure at such temperature renders the blood inert for immunological purposes. In place of heat, toluol or 50 per cent glycerin can be used to destroy the pathogenicity of the organism. Further, it has been found that blood from affected birds loses its virulence after 48 hours and can then be employed for the immunization of susceptible birds.

BORRELIA THEILERI (SPIROCHAETA THEILERI, SPIRILLUM THEILERI)

In 1902 Theiler[3] found in the blood of cattle, a long, spiral organism which is capable of producing a febrile disturbance of minor importance. Theiler and others have demonstrated the organism to be transmitted by ticks, particularly Rhipicephalus decoloratus and Rhipicephalus evertsi. According to Theiler, the disease produced by this organism is similar, in some respects, to piroplasmosis but much more benign.

Subsequent to the identification of the organism in cattle Theiler observed in sheep a similar spirillum which he considered identical with that found in cattle. Martoglio and Carpano[4] also apparently found the same organism in sheep and designated it "Spirochaeta ovis" or "Spirillum ovis." Then Stordy[5] found in the blood of a sick horse in East Africa a spirillum which has been designated "Spirochaeta equi." Investigation tends to indicate that this organism, like those found in sheep, is identical with Borrelia theileri.

Borrelia theileri is an actively motile organism, measuring 20 to 30 microns in length and 0.25 to 0.30 micron in thickness. Like Borrelia gallinarum, the spirals are not close together. It can be stained with carbol-fuchsin, gentian-violet and Giemsa stain. It has not been cultivated.

[3] Theiler, Jour. Comp. Path., xvii, 47, 1904.

[4] Martoglio and Carpano, Ann. d'Igrene Sperim., xiv, 1904.

[5] Stordy, Jour. Comp. Path., xix, 1906.

BORRELIA HYOS (SPIROCHAETA HYOS)

King and Baeslack,[6] in 1912, published a report of the observation of a spirochaete in the blood of hogs suffering from hog cholera. They were of the opinion that the organism was of etiological significance. Subsequent investigations have shown that such organism is not the causative factor of hog cholera.

Borrelia hyos is a short, thick spiral, the average measuring 5 to 7 microns in length and about 1 micron in thickness. The organism possesses a flexible body with blunt, rounded ends. It rotates actively about its longitudinal axis. It can be best stained by Levaditi's silver impregnation method and can also be well demonstrated by the India ink method.

The organism is a strict anaerobe and can be cultivated under proper atmospheric conditions in blood broth and acitic broth.

In connection with Borrelia hyos, King, Baeslack and Hoffman[7] observed in blood from hogs suffering from hog cholera, granular bodies which they were inclined to believe might be a stage in the development of the organism.

[6] King and Baeslack, Jour. Inf. Dis., xii, 39, 1913.

[7] King, Baeslack and Hoffman, Jour. Inf. Dis., xii, 206, 1913.

PART V

PATHOGENIC FUNGI

CHAPTER XXIX

Blastomycetes and Hyphomycetes

In the vegetable kingdom there is recognized a large group of lower forms of plant life known as "*Thallophytes*." This group is made up of those plants which do not possess stems, leaves or roots. Thus, it includes the bacteria, the various algae and the fungi. In bacteriology it has been a rather common practice, in grouping such of the Thallophytes as are of interest, to consider three principal groups, the *Lower Bacteria* (Eubacteriales—true bacteria), *Higher Bacteria* (Trichomycetes) and the *Fungi* (Blastomycetes, Hyphomycetes).

The higher bacteria have been considered as occupying an intermediate position between the lower bacteria and the fungi, being more specialized than the former but not quite so complex as the latter. Under the system of classification followed in this work, the pathogenic higher bacteria have been grouped under the Orders other than Eubacteriales and the various families, genera and species of importance in veterinary medicine have already been dealt with. Thus, we have left for consideration only the pathogenic fungi, a group of organisms producing various types of *mycoses* in man and the lower animals.

The fungi constitute a vast group ranging from the unicellular yeasts to the more complex multicellular forms such as mushrooms and toadstools. Of the innumerable types of fungi there are at present but two groups containing disease-producing members of importance in veterinary medicine. These are the *Blastomycetes* (yeasts) and the *Hyphomycetes* or *Eumycetes* (moulds).

Blastomycetes or yeasts ordinarily are unicellular, round, oval or more elongated bodies which reproduce chiefly by a process commonly termed "*budding*." In this process the nucleus of the parent cells divides and at about the same time the cell throws out on its margin a projection or "bud." A portion of the nucleus goes into the bud which rapidly develops into a new cell having the characteristics of the mother cell from which it separates at the constriction. Frequently, in the reproduction of some of the blastomycetes, it will be noted that following multiplication the mother and daughter cells do not always separate immediately but continue in contact, becoming considerably elongated. As the process of reproduction is repeated under such circumstances a mycelium-like structure results. In this respect such yeasts approach the characteristics of the Hyphomycetes.

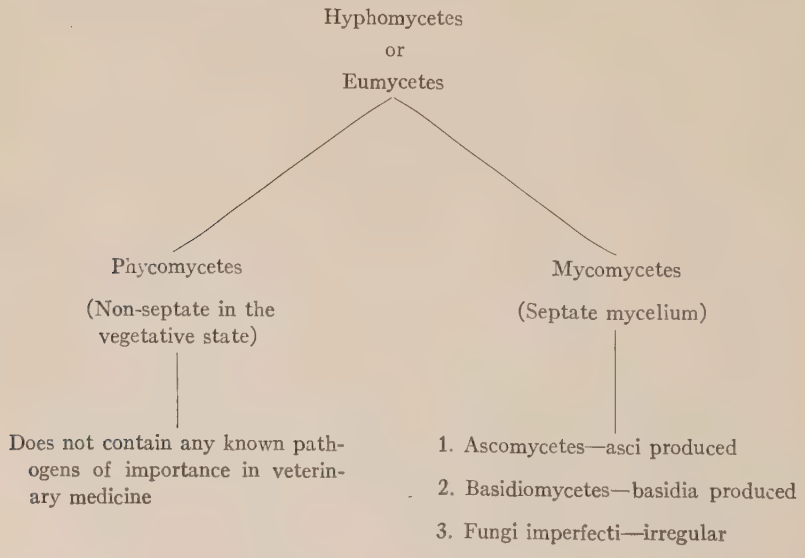
Aside from reproduction by budding some types of blastomycetes pro-

duce spores (*ascospores*), each cell, depending upon the species, producing from two to eight. The yeast cell containing spores is known as the "*ascus*." The formation of ascospores by yeasts is another factor indicating close relationship to the higher fungi.

Those blastomycetes which are definitely known not to produce spores are, by some authorities, placed in a genus designated "*Torula*."

The Hyphomycetes or moulds are the most complex of the plant organisms considered in bacteriology. They differ from the lower structures in that they are multicellular and form a network or *mycelium* of branching filaments known as "*hyphae*." When propagating, growth proceeds from the end of the hypha opposed to that which was originally the spore and continues in such manner. In others words, unlike the true bacteria which grow from both ends and divide in their middle portion, the hyphomycetes grow out but from one end of the hyphae. In some species of Hyphomycetes the hyphae are not distinctly separated but form a continuous mycelium (*non-septated*) with multiple nuclei. In other forms the individual hyphae are separated by *septa* (Fig. 59).

The classification of the moulds is far from satisfactory. From our standpoint, however, there are only a few of known importance so the classification is not of very great consequence. We can conveniently place them in two groups, the *Phycomycetes* and *Mycomycetes*. The pathogenic forms are included in the latter group, the Phycomycetes being of little significance insofar as the purpose of this work is concerned. From a botanical viewpoint such grouping as this would by no means be sufficient but from a bacteriological standpoint it will suffice.



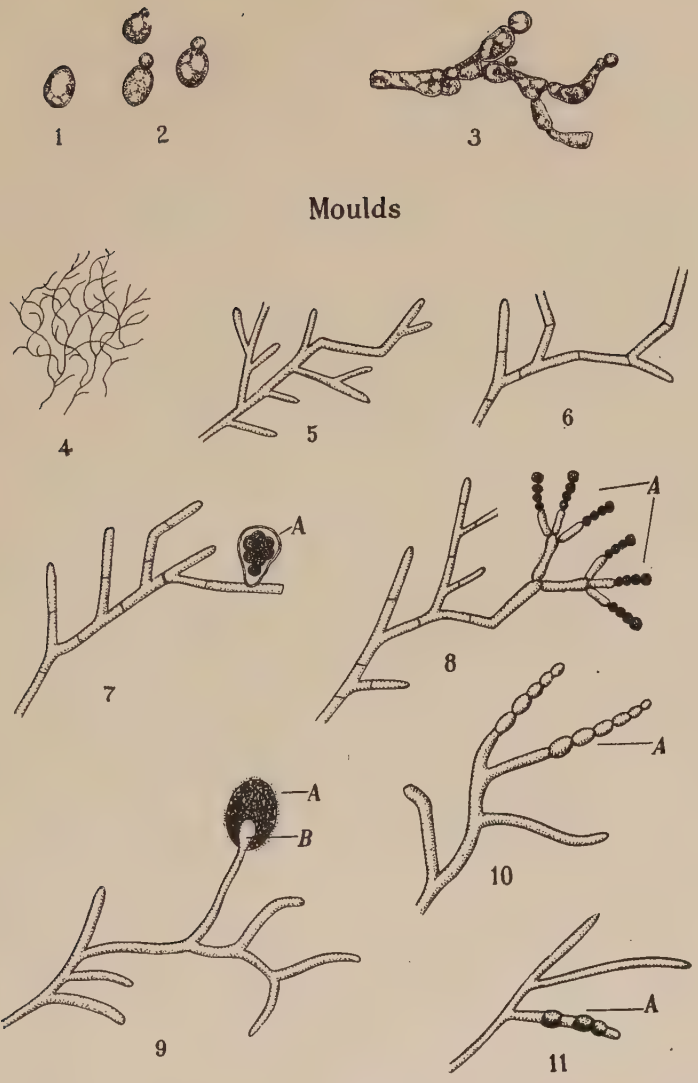


FIG. 59

Yeasts: 1, yeast cell; *2*, budding yeast cells; *3*, blastomyces forming mycelium-like structure.

Moulds: 4, mycelium; *5*, non-septate mycelium; *6*, septate mycelium; *7*, (*A*) ascus containing ascospores; *8*, hypha branched to form two conidiophores each of which has given rise to three sterigmata on the ends of which are the conidia; *9*, (*A*) sporagium containing spores, (*B*) columella; *10*, (*A*) conidia formed by simple constriction of hyphae; *11*, (*A*) chlamydospores.

The Phycomycetes do not develop a basidium or ascus. In the vegetative state they are non-septate organisms. Reproduction in some species, however, occurs through the formation of spores in a *sporangium*, in other species *conidia* and *chlamydospores* (spore surrounded by a heavy capsule) are developed. In cases where a sporangium is produced the terminal end of the particular hypha swells and becomes separated from the rest of the stem by a straight or curved septum. In this sporangium the spores develop. That portion of the hypha supporting the sporangium is designated the *columella*.

The Mycomycetes are septate organisms which may be further divided into three groups. The first group is *Ascomycetes* or *sac fungi* which form *spore sacs* or *asci*. The second group is the *Basidiomycetes*, members of which produce a *basidium* which carries the *basidiospores*. The third group is a large one known as "*Fungi imperfecti*." This group includes a vast number of fungi which cannot be satisfactorily classified.

In the case of the Ascomycetes the fruiting hypha becomes swollen at its terminal end to form a sac in which a definite number of spores, varying with the species, develop. The sac or ascus finally ruptures at its tip releasing the spores which later develop into new organisms.

The basidium which characterizes the Basidiomycetes is a finger-like type of sporophore. On this basidium there develops a definite number of *conidia* (asexual spores), the number commonly being eight although it varies according to the species. There are no members of this group of importance in veterinary medicine.

When considered from the standpoint of method of reproduction the Fungi imperfecti are more or less rudimentary types which do not possess distinctive fruiting bodies.

CHAPTER XXX

Pathogenic Blastomycetes

Epizootic lymphangitis of equines, caused by Blastomyces farciminosus, is the only type of blastomycosis of any great importance in veterinary medicine. Occasionally one may find reference to other types of blastomycosis in the lower animals but they are rare and variable in character. The author has observed a type of blastomycotic dermatitis in dogs which was subsequently transmitted from the dog to two members of the owners' family.

BLASTOMYCES FARCIMINOSUS (CRYPTOCOCCUS FARCIMINOSUS, SACCHAROMYCES FARCIMINOSUS)

In 1873 Rivolta demonstrated in the pus from affected lymph nodes in cases of epizootic lymphangitis, the organism which we now designate "Blastomyces farciminosus." Rivolta's observation was soon confirmed by other investigators but the organism was not cultivated until about 24 years subsequent to its discovery. Tokishige,[1] working with epizootic lymphangitis in Japan, was the first to propagate the organism in pure culture.

Epizootic lymphangitis, in countries where it is prevalent, is of considerable importance. The disease, once it becomes well established, runs a long, chronic course. It is exceedingly obstinate and does not yield readily to treatment. The malady occurs in France, England, Egypt, South Africa, Italy, Japan, Russia, India and the Philippine Islands. There are several records of its occurrence in the United States.

The malady is characterized by a purulent inflammation of the superficial lymph vessels and glands. It commonly involves the lymphatics of the legs, chest wall and neck, other parts of the body being less frequently attacked. In extensive cases the entire body may become involved. The infection is apparently introduced into the tissues through superficial injuries, harness or saddle chafing, etc. The affected lymph vessels become swollen and painful, standing out as tortuous strands connecting the enlarged nodes. When the nodes rupture or are incised, a thick, creamy, yellowish-white pus is discharged. At the site of the ruptured node a chronic, easily-

[1] Tokishige, Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, xix, 105, 1896.

bleeding ulcer is formed. Blastomyces farciminosus can be readily demonstrated in the pus from the affected lymphatics.

Man possesses considerable resistance to this organism but cases of infection in the human family have been recorded.

One or two investigators have questioned the classification of Blastomyces farciminosus as a yeast, maintaining that it is protozoan in nature. The author has spent a large portion of the past year (1926) studying epizootic lymphangitis of horses in the Philippine Islands. The organism has been studied as it occurs in pus and in the tissues and it has been cultivated. As a result of these observations I have no hesitancy whatever in agreeing that it has been properly identified as a vegetable organism belonging to the Blastomyces, and that it is the primary etiological factor of the disease commonly known as epizootic lymphangitis.

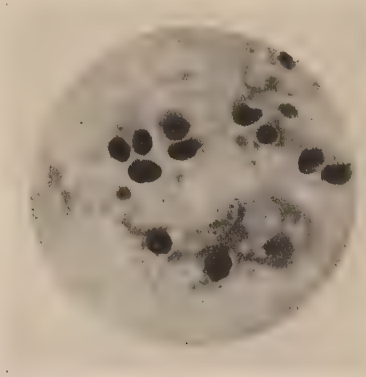


Fig. 60. Blastomyces farciminosus (Pus from Lymph Node)

Morphology and Staining Characteristics. Blastomyces farciminosus in pus or tissue preparations appears as a doubly contoured, oval cell, 3 to 4 microns in length and about 2.5 to 3.5 microns in breadth. Budding processes are present on some of the cells in such preparations. The content of the cell may be perfectly homogeneous or it may appear granular. Cultures of the organism reveal single cells and cells arranged in filaments (see fig. 61).

The organism can be readily demonstrated in unstained as well as stained specimens. It is not readily stained by all of the ordinary aniline dyes. Diluted carbol-fuchsin applied to the preparation with slight heating gives satisfactory results. In the author's experience the Gram-method gave excellent results. Acetone is used for decolorization and is applied for

only a very brief period of time. The pus cells, tissue debris, etc., are stained red with the safranin counterstain while the blastomyces retain the gentian-violet. If the decolorization process is "pushed" the organisms frequently appear Gram-negative.

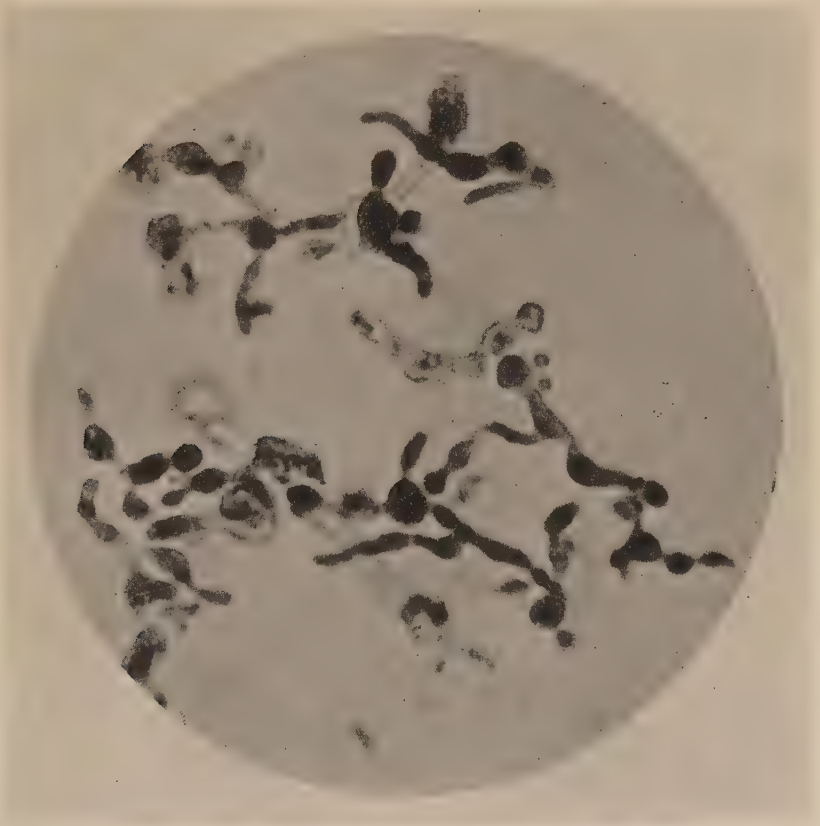


FIG. 61. BLASTOMYCES FARCIMINOSUS (CULTURE)

Cultural Characteristics. Blastomyces farciminosus is an aerobic organism which, under present methods of cultivation, grows exceedingly slow. In numerous instances growth fails entirely. Growth cannot ordinarily be detected until after 20 to 40 days. The organism is, apparently, not fastidious in its temperature requirements as there is no appreciable difference in the time required for propagation when the cultures are left at room temperature and when kept in the incubator at 37.5°C.

Agar. The growth is very slow on agar, frequently not succeeding at all. When colonies do develop they appear as small, grayish-white granules which slowly enlarge, become tough and take on a folded appearance which has been likened to the convolutions of the intestines.

Gelatin. Gelatin is even less favorable for the growth of Blastomyces farciminosus than agar. Six or seven weeks ordinarily elapse before even a very small percentage of cultures show evidence of growth. Colonies on gelatin are slightly yellowish in color and of a sandy or granular appearance. The medium is not liquefied.

Bouillon. In broth, after several weeks, the organism develops as a white, flaky growth which collects at the bottom of the tube. Serum added to the broth favors growth.

Litmus Milk. This medium is unsuited for the propagation of this organism.

Potato. Potato medium is somewhat more satisfactory for the growth of Blastomyces farciminosus than most of the common media. Growth occurs earlier in some cases but in others the period of incubation is not materially shortened. As is the case with the various other media growth frequently fails entirely. The growth on potato is of a yellowish or light brown color.

Egg Medium. In growing Blastomyces farciminosus the writer has obtained best results with egg medium. Growth occurs earlier and is more luxuriant than with the other types of media employed. The colonies are finely granular and of a light yellowish color. Later the colonies become larger and wrinkled.

Fermentable Media. Blastomyces farciminosus does not produce acid or gas from the ordinary sugars commonly employed in bacteriology.

Resistance. Like most of the organisms of this group Blastomyces farciminosus is a very resistant organism. It will tolerate drying, high temperatures and disinfectants for rather long periods of time.

Pathogenicity. Guinea pigs inoculated in the inguinal region occasionally develop lesions in the regional glands. The process in such animals is of a very low grade nature and never becomes generalized. Inoculation of rabbits occasionally results in the formation of an abscess at the point of inoculation.

CHAPTER XXXI

Ascomycetes

Some authors include certain of the yeasts (blastomycetes) in this class. In the present work, however, the blastomyces have been separated from this group, although, as previously indicated, asci-producing yeasts are very closely related to the Ascomycetes.

The Ascomycetes include several genera of importance in veterinary medicine. These are Trichophyton, Microsporum, Achorion and Aspergillus. In addition a fifth genus known as "Penicillium" might be merely mentioned. This latter genus is made up of non-pathogenic members but is of passing interest because species of Penicillium are frequently found on mouldy meat, cheese, etc., and, hence, are met with in the inspection of meat and dairy products.

GENUS TRICHOPHYTON

The species making up this genus are responsible for herpes or ringworm in man and animals. In the lower animals ringworm is most common in cattle while horses are next in order of frequency of occurrence. The disease is much more rare in dogs, cats, sheep and swine.

There has been considerable confusion as to the species responsible for ringworm in the lower animals. In man Trichophyton tonsurans is the common variety. Some authors consider this same species commonly responsible for herpes in the lower animals. Others maintain that there are varieties and sub-varieties of Trichophyton tonsurans and that the etiological factors of the common ringworms of lower animals are to that extent removed from the tonsuran species.

Various French investigators divided the genus Trichophyton into two groups—Trichophyta megalospora (large "spores") and Trichophyta microspora (small "spores"). Others have created a separate genus known as "Microsporum," the members of which consist of small spores but, in addition, differ in their arrangement from members of the genus Trichophyton. Although the relationship is close, we recognize the genus "Microsporum" as distinct from Trichophyton, and, in this work, will consider it separately.

We use the term "Trichophyton megalospora" to designate those ringworm fungi which form spores (really segments, not true spores), 4 to 8

microns in size, which occur in chain formation in and around the hair. Where such spores are smaller in size (2 to 4 microns) and similarly arranged in chains we term them "Trichophyton microspora."

The genus Trichophyton is also divided into two groups according to whether the parasite is found only inside of the hair or both inside and around it. Where found only within the hair it is known as an "*endothrix*." When it occurs both inside and outside of the hair it is designated an "*endo-ectothrix*" or simply an "*ectothrix*."

For the demonstration of the ringworm fungi the following procedure gives satisfactory results:

Obtain the specimens from the periphery of the most recent lesions. Use

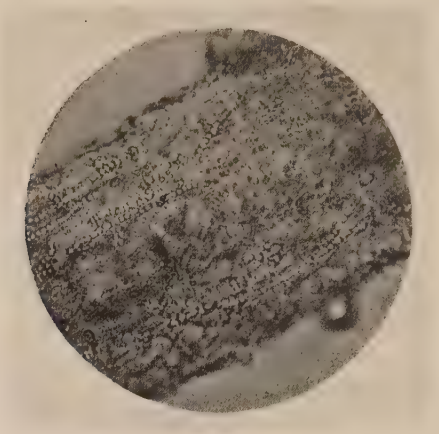


Fig. 62. Trichophyton equinum (Section of Hair)

a sharp scalpel and take the scrapings deep enough to insure getting some hair roots. Place the specimen in a test tube or Petri dish and add chloroform or ether to it to remove such fat as it contains, allowing it to remain in the fat solvent for one or two hours or even overnight. Pour off the chloroform or ether and place a small amount of the specimen on a slide. Cover it with a few drops of 10 per cent sodium hydroxide and warm it gently over a flame. Be sure to use sufficient of the sodium hydroxide solution to insure against the specimen going dry. Place a cover-glass over the specimen and with gentle pressure flatten it out. The warm sodium hydroxide will have made the hairs and tissue fragments transparent, thus making a satisfactory examination possible.

For the cultivation of the fungi Sabouraud's maltose agar can be used to good advantage. The formula for this is given in the section on Culture Media and Their Preparation. Potato is also a good medium for a number of fungi.

Pure cultures are exceedingly hard to isolate because of the numerous contaminating organisms present in the average specimen. In attempting to obtain pure cultures the method recommended by Kitt can be employed. The material to be cultured is treated with 10 per cent sodium or potassium hydrate for 5 or 10 minutes, washed with sterile water and then plated. The ringworm fungi are quite resistant to alkalies. A temperature of 30° to 33°C. is best suited for this group of organisms.

Trichophyton equinum

Trichophyton equinum is of the megalosporon, ectothrix variety of ringworm fungi and is a common cause of the disease in the horse. It is also pathogenic for man.

The spores are from 4 to 6 microns in size.

The organism will propagate readily on maltose agar producing a so-called "orbicular" growth. The growth at first is white, later becoming yellowish and in old cultures reddish in color.

Trichophyton mentagrophytes

This organism belongs to the Trichophyta microspora group and the ectothrix variety. It produces ringworm in horses, cattle, dogs and possibly sheep and swine. Man can also be infected with this species.

On Sabouraud's agar the growth appears white and powdery with tapering projections at its periphery.

Trichophyton granulosum

This species is closely related to Trichophyton mentagrophytes. It is the cause of a trichophytosis of the horse characterized by the development of very numerous, minute patches in which the hair mats together, later falling out leaving small, bare areas.

On maltose agar discoid, frequently umbilicated, powdery, yellowish-white colonies develop. These colonies have large granular prominences dotting their surfaces.

Man can be infected with this species.

Trichophyton felineum

Blanchard, in 1895, classified this organism under the above heading. It is also known as Trichophyton niveum (Sabouraud) and Trichophyton radians (Sabouraud, 1909).

Trichophyton felineum is a megalosporon, ectothrix variety causing a pustular ringworm in cats. Horses, cattle, sheep, dogs and swine may occasionally become infected with this species. Man is also susceptible to it.

On Sabouraud's maltose agar umbilicated, white, powdery colonies with radiating projections at their periphery develop.

Trichophyton megnini

This is a megalosporon, ectothrix type of Trichophyton, capable of producing ringworm in fowls. It is also reported as being occasionally infectious for man.

The spores of this organism are 8 or 9 microns in diameter.

On maltose agar white, velvety colonies appear. These later become pinkish in color.

GENUS MICROSPORUM

The genus Microsporum is made up of organisms which develop small spores or segments (2 to 3 microns) but in place of being arranged in the chain formation which characterizes Trichophyton microspora, they are found closely grouped about the hair in an irregular, mosaic-like manner. The white, mouldy covering thus formed around the hair extends several millimeters above the epidermis.

Microsporum minimum

This species of Microsporum is closely related to Microsporum audouini, a very common parasite of the human family. It is frequently found as the causative agent of one type of ringworm in horses, particularly foals. Man is also susceptible.

The growth on maltose agar is of a white, furrowed character. Unlike most of the microsporons, the fine duvet is either entirely lacking, or is present in scanty amount, is short and of a light pink color.

Microsporum lanosum

Microsporum lanosum is another species very closely related to Microsporum audouini. It is commonly found in ringworm of dogs. Some

authors classify it as "Microsporum canis" while others consider it merely a variety of the species audouini and designate it "Microsporum audouini, variety canis."

In addition to its pathogenicity for the dog this fungus is also capable of producing ringworm in the horse and possibly other animals. Further, it is occasionally responsible for a severe form of tinea capitis and sometimes tinea barbae and corporis in the human family.

The mosaic sheath surrounding the hairs in ringworm due to this organism, is composed of somewhat rounded, or polyhedral spores of from 2 to 3 microns in diameter.

On maltose agar the growth of Microsporum lanosum is rather slow in starting but after commencing it becomes abundant. At first the colony is downy and plaque-like. After 3 or 4 weeks the central portion becomes umbilicated with the periphery forming a velvety ring covered with a fine, snow-white duvet. As the culture continues to age the duvet becomes yellowish.

Microsporum felineum

This species of Microsporum has been reported as fairly common in the cat in England, Belgium, the United States and Canada. It can be transmitted to the dog and possibly other species including man.

Morphologically it does not differ from the common members of the genus. On maltose agar an abundant growth occurs. The colonies are disc-like, flat and covered with a grey duvet.

GENUS ACHORION

Fungi making up the genus Achorion are, in many ways, closely related to members of the genera Trichophyton and Microsporum. Organisms of the Achorion genus are responsible for the disease known as "favus." In veterinary medicine favus is probably best known as it occurs in chickens. The disease, however, is not limited to fowls but is occasionally seen in dogs, rabbits, cats, mice and rats. In certain localities it is exceedingly common in mice. Man is frequently infected with favus fungi of animal origin, particularly that of mouse favus.

In favus, minute, cup-shaped, sulphur-yellow crusts or scabs known as "scrutula" develop around the hair at the mouth of the follicle.

Organisms of the Achorion genus, when living a parasitic existence, are frequently found to develop mycelial segments longer than those of members of the Trichophyton and Microsporum genera.

Achorion schoenleini

Achorion schoenleini is the common etiological factor in favus of man. This same species or variety is also capable of producing favus in cats and rabbits and possibly other animals.

In affected animals the organism can be demonstrated in large numbers by macerating several scrutula in a little sodium hydroxide solution. The mycelium of long, frequently branching hyphae, is often so dense that it forms a felt-like mat. Scattered through the close net-work of mycelium, spores of from 3 to 5 microns in diameter are observed. On close examination it will be noticed that the branching segments frequently terminate in a single row or filament of spores.

Achorion schoenleini can be readily grown artificially. It grows much better on maltose agar made by adding maltose and agar to standard beef infusion broth than on the medium of Sabouraud. Its optimum temperature requirement is about 30°C. The culture develops as a tough, waxy-appearing, yellowish, convoluted or "cerebriform" growth. Subsequently the growth loses its wax-like appearance, becoming covered with a grayish-white, powdery duvet. Achorion schoenleini will grow quite satisfactorily on potato. On such medium the growth is definitely raised, hilly and of a white color, later becoming grayish-yellow.

In cultures, double-contoured terminal structures referred to as "yellow-bodies" or "favus bodies" are frequently observed. These structures are somewhat pear-shaped, about 8 to 12 microns at their greatest diameter, and contain a granular material.

Achorion schoenleini liquefies gelatin in a comparatively short time. It is more rapid in this respect than the members of the Trichophyton genus.

Achorion gallinarum (*Lophophyton gallinarum*, *Epidermophyton gallinarum*)

This organism is the common cause of favus of chickens. In the beginning the disease usually involves the comb, wattles and unfeathered skin around the eyes. Minute, mould-like, grayish-white scrutula appear and as the process develops these coalesce covering practically all of the involved comb, wattles, etc. Subsequently the feathered skin of the neck, back and other parts of the body is attacked. When this occurs the feathers of the affected region become surrounded by a thick, scaly scrutulum, become loose in their follicles and drop out.

Two forms of Achorion gallinarum occur in the lesions. One form consists of long, slender, tortuous filaments showing but little protoplasmic

substance. In the other form the hyphae are short, thicker, branching and possess a protoplasm containing refractile granules.

Achorion gallinarum develops more slowly on artificial media than some of the other species. The colony is raised, ringed and convoluted. It develops a bright red color which serves to distinguish it from other species.

This fungus is capable of producing tinea-like lesions in man.

Achorion quinckeanum

Achorion quinckeanum is the etiological factor of the common favus of mice. Rats are also occasionally infected with this fungus. It is readily transmitted to man.

In the lesions the organism is observed as a mycelium consisting of filaments which, to a large extent, are made up of small, spore-like segments.

When grown artificially the colonies are of a grayish-white color and quite downy.

Achorion canis (*Oöspora canina*)

Favus in dogs is frequently the result of infection with a fungus which is classified as a member of the achorion genus by some and by others as belonging to the genus "Oöspora." The organism differs from the ordinary members of the Achorion genus in that it multiplies exclusively through the formation of oöspores.

GENUS ASPERGILLUS

The fruiting hyphae of the members of this genus are not ramified but terminate in rounded or oval heads from which a number of claviform sterigmata project in all directions. These sterigmata each bear a chain of conidia.

As a general rule the organisms of this genus live a saprophytic existence, and are chiefly of importance in that they are troublesome as contaminants about the laboratory. Occasionally, however, some species are pathogenic. When found as disease-producers they commonly produce a pneumomycosis or mycotic pneumonia. In birds pneumomycosis or "aspergillosis" is not an uncommon malady. It is probably found most often in pigeons although chickens, ducks, geese, parrots, canaries, etc., are susceptible. Rarely, mycotic pneumonia is observed in horses, cattle and sheep. The disease is also occasionally observed in man.

Aspergillus fumigatus

This species of aspergillus is the one most commonly found in pneumomycosis. The fungus is very frequently found on hay, straw, grain, etc.

Aspergillus fumigatus can be readily demonstrated in the affected tissue and can also be artificially cultivated from same without difficulty. In the tissues the characteristic fruiting hyphae or conidiophores and bluish-green spores can be demonstrated, provided free oxygen has been available in air passages and pulmonary alveoli. Spores are not produced in the absence of oxygen. When grown on slightly acid media bluish-green colonies develop. These later take on more of a grayish color. If the medium employed is of an alkaline reaction the colonies may be of a brownish color rather than green.

The ordinary mycelial filaments measure from 2 to 3 microns in thickness. The fruiting hyphae are thicker averaging from 4 to 5 microns in breadth and the enlarged oval or round terminal portion varies in diameter from 10 to 20 microns. The conidia average 2 to 3 microns in diameter.

Aspergillus fumigatus is a very tenacious organism. It will resist high temperatures over long periods of time and is not readily destroyed by reasonable exposure to antiseptic solutions. Autoclaving for 20 minutes at 15 pounds steam pressure readily destroys it.

Aspergillus nigrescens

While not found as frequently as the foregoing fungus, Aspergillus nigrescens is occasionally observed as the etiological factor in mycotic pneumonia of the lower animals.

This species differs from Aspergillus fumigatus in that the conidia are dark brown or black, giving the growth a chocolate color.

CHAPTER XXXII

Fungi Imperfecti

This class includes a great group of organisms, the complete life-histories of which are not definitely known. Some authors include in this group so-called "higher bacteria," certain of the blastomyces, etc. Under some systems of classification most of the pathogenic fungi are placed under Fungi imperfecti. In this work we will briefly refer to three genera which we have grouped under this heading. These are Oidium, Sporotrichum and Fusarium.

GENUS OIDIUM

Members of the genus Oidium possess very simple sporophores. The hyphae are sometimes long and branched while, on the other hand, poorly developed hyphae are observed. Spores are formed by constriction of the hyphae at their extremities. Chlamydospores are observed in some species. Besides typical mycelial forms, round or oval, yeast-like cells showing evidence of budding are observed.

Odium albicans (*Monilia albicans*, *Endomyces albicans*, *Monilia candida*, *Saccharomyces albicans*)

Oidium albicans is the cause of a rather common form of thrush in young children. An analogous type of stomatitis is occasionally observed in birds, lambs, calves, etc.

The disease in the lower animals is characterized by the development of grayish-white or yellowish patches on the mucous membranes of the mouth and fauces and in birds such lesions are also found in the crops. Subsequently pseudo-membranes are noted. The lesions are not surrounded by an inflammatory zone, a point which differentiates thrush from some other forms of stomatitis.

Oidium albicans can be demonstrated in material from the lesions in the form of slightly branching filaments of various size and also as single yeast-like cells. The filaments are observed to be divided into segments which contain vacuoles and granules.

The organism can be cultivated easily on media of an acid reaction. It develops more readily on solid than in fluid media. The colonies are white, round and waxy with a fine, granular surface. The organism grows

exceedingly well on turnip medium. Such medium is prepared in the same manner as potato medium. It w ll grow well at room temperature but develops more rapidly at 37.5°C. The fungus will grow on gelatin without liquefying it. The yeast-like forms are more abundant when the organism is grown on media containing carbohydrates.

GENUS SPOROTRICHUM

Members of this genus reproduce through the development of true conidia but true conidiophores are absent. A mycelium of slender, branching hyphae occurs with the conidia attached to the sides and ends of the segments by short sterigmata.

Sporotrichum beurmanni

Sporotrichum beurmanni is the most common cause of human sporotrichosis, a chronic affection characterized by the formation of gummatous-like swellings in the subcutaneous tissues and involvement of the lymphatics.

From a veterinary standpoint this fungus is of interest because it, or a variety of the same species, is capable of producing a type of lymphangitis in horses.

Careful examination of material from lesions will reveal the presence of the yeast-like forms only. It is frequently difficult to satisfactorily demonstrate them. They occur as oval, rounded, or short, rod-shaped forms 3 to 5 microns long and 2 to 3 microns in thickness. A number of them will be found phagocytized.

The organism grows well on Sabouraud's maltose-agar, growth starting to develop within 5 or 6 days at room temperature. In the beginning the colony appears as a small, grayish-white, fringed structure. As it develops the center becomes raised, wrinkled, and takes on a buff or dark-brown color, in some instances even becoming black. In old cultures the glistening appearance of the colonies is frequently lost, the growth becoming dry and powdery.

The organism will grow satisfactorily on glucose-gelatin, slowly producing liquefaction of the medium. Saccharose is split with the formation of acid but no gas. Stained by Gram's method the organism is Gram-positive.

Microscopic examination of cultures of Sporotrichum beurmanni will reveal mycelial threads measuring approximately 2 microns in diameter. A rather large number of small, round or oval conidia are found attached by short sterigmata to the sides and ends of the hyphae.

Sporotrichum beurmanni is pathogenic for rats and mice.

Sporotrichum schencki

This organism is similar in many respects to Sporotrichum beurmanni. Some authors consider them identical. There is evidence, however, which tends to indicate that they are different species. Sporotrichum schencki, unlike the preceding organism, possesses little or no pathogenicity for rats and mice. Then, from a cultural stand-point, conidia production is much more limited than with Sporotrichum beurmanni.

It is possible that some cases of sporotrichosis in the lower animals are due to this species.

GENUS FUSARIUM

Members of this genus produce a fine, loose, mycelium with numerous septa not widely separated. Conidia are produced at the ends of conidiophores and those characteristic of the genus are sickle-shaped and rather large, measuring 25 to 50 microns in length and 2.5 to 5 microns in width.

Fusarium equinum

Nörgaard and later Melvin and Mohler described this fungus as the etiological factor in a type of dermatitis of horses. The disease is characterized by an intense pruitis and a scurfy condition of areas of the skin with subsequent loss of hair. Preparations from affected areas will demonstrate the mycelial threads and spores.

The organism can be readily cultivated on artificial media. Potato is an exceptionably good medium for its propagation. The colonies appear white and frost-like. Microscopically three types of spores are observed —*microconidia, macroconidia* and *chlamydospores*. The microconidia, as the name implies, are small, usually oval in shape and occur singly and in pairs. The macroconidia are the large, sickle-shaped structures (25 to 50 by 2.5 to 5 microns) typical of the genus. The chlamydospores form within the mycelial segments, becoming walled off.

In Dunham's peptone solution the under portion of the surface growth assumes a chocolate color.

PART VI

THE PROTOZOA

CHAPTER XXXIII

The Parasitic Protozoa. Their Mode of Life and Relation to Their Hosts. Some Technical Considerations[1]

The fact that the study of bacteria in their relation to disease involves an excursion into the field of cellular biology makes it desirable that a work on bacteriology should include at least a brief treatment of the corresponding animal forms—the Protozoa. This is particularly important because parasitic protozoa, and their hosts, react to each other, for the most part, in a manner quite different from the reactions observed in bacterial invasions. Moreover, the Protozoa are a potential source of confusion to those who have had no opportunity to study them, but who are likely to encounter them in the pursuit of other work. Tissue cells, atypical or degenerated nuclei, and cell inclusions of obscure origin frequently have been described as "coccidia" or "amœbæ" and the writer recalls one instance where an elaborate paper was published on a flagellated parasite of one of the lower animals, it later being discovered by another worker that the "flagellates" were spermatozoa. Of course, such tragic errors may quite as well arise through lack of knowledge of the normal histology of the host as from a corresponding lack of knowledge of protozoa, but most of them may be avoided if one bears in mind a few fundamentals concerning the morphology and physiology of the Protozoa. An attempt to set forth some of those principles will be made in this chapter.

The protozoa are loosely defined as "unicellular animals." However, there still is some diversity of opinion as to whether such a definition is either adequate or accurate. It is open to question whether a protozoön in the trophic stage of its life cycle may correctly be compared to a tissue cell. In the first place an actively feeding and reproducing protozoön, if we are to consider it as a cell, is one showing physiological balance to a degree quite comparable with that of a metazoan organism. It is capable of both self-support and reproduction. On the other hand, while there is a certain structural resemblance, the epithelial or muscle cell is distinctly unbalanced physiologically in its relegation to some special function. It must receive its nourishment from other sources.

[1] For this chapter the author is greatly indebted to Prof. Frank G. Haughwout, Protozoölogist of the Bureau of Science, Manila, P. I.

This has led Dobell[2] to discuss the Protozoa as non-cellular rather than unicellular animals. Other authorities speak of the entire series of changes comprehended in the protozoan life-cycle as forming the basis of comparison between Protozoa and Metazoa. One common definition of the Protozoa, however, can provoke no controversy whatever. They frequently are spoken of as "the simplest forms of animal life." No one who ever has seriously studied these fascinating organisms can regard them in this light. The most primitive forms of fresh water amœbæ hold secrets that have defied the keenest and most experienced investigators of the group.

Protozoa defined. Calkins[3] has formulated an answer to the question "What is a protozoön?" that is exceedingly useful because its comprehensiveness lays down the foundation of just what it is intended to set forth in this chapter. The definition is as follows:

> A protozoön is a primitive animal organism usually consisting of a single cell, whose protoplasm becomes distributed among many free living cells. These reproduce their kind by division, by budding, or by spore formation, the race thus formed passing through different form changes and the protoplasm through various stages of vitality collectively known as the life cycle.

This definition may now be analyzed.

Significance of the Life Cycle. The Protozoa are primitive in the sense that their structure as compared with the Metazoa is relatively simple. They have no organic, circulatory or nervous systems as those things are seen in the higher animals. They are, however, equipped to take in, digest and assimilate food in one form or another, many of them possess a neuro-motor complex which may be likened to a rudimentary nervous system, all are capable of reproduction and some pass through processes of fertilization, similar in all essentials, to those occurring in the Metazoa. They are considered as animals, because the animal type of nutrition prevails among them. It is the view of the writer that all unicellular organisms that derive their nourishment from photosynthetic processes should be considered to be plants.

Usually, the Protozoa are encountered as single cells, but in certain processes of reproduction one may encounter groups of cells more or less closely attached, while other Protozoa characteristically form colonies.

In the process of reproduction, and this varies greatly among the Protozoa, the original cell loses its individuality except in cases where the

[2] Dobell, C. Clifford, "The Principles of Protistology," Arch. f. Protistenk., xxiii (1911), 269–310.

[3] Calkins, Gary N., Protozoölogy, (1909), p. 17, Philadelphia, Lea & Febiger.

parent cell survives following a process of budding or gemmation. The process of division proceeds in geometrical progression, the protoplasm of the original cell being truly distributed among many free living cells.

Reproduction may be by binary fission by which the parent cell divides to form two daughter cells that resemble the parent cell; or by multiple fission (styled schizogony in certain sporozoan forms), in which the parent cell divides to form more than two daughter cells. Budding involves the separation from the parent cell of buds of nucleated cytoplasm, that in time grow to resemble the parent cell which may or may not survive. Budding may be internal or external. Spore formation (*sensu strictu*) is an elaborate process, accompanied by fertilization and the encapsulation of the organism in a resistant cyst or spore case. It is not to be confused with simple encystment which is unaccompanied by processes of fertilization.

Form changes within the life cycle offer the greatest difficulties in dealing with new species. The dictum of Schaudinn "Die Kenntniss der Entwickelung ist das erste Postulat der Protozoenforschung," is fundamental. No species of protozoön may be classified accurately until its complete life history has been worked out. It is true that many species, both parasitic and non-parasitic, are relatively monomorphic, but others present a bewildering series of form changes in the course of their life cycle. Some amœbæ pass through a flagellated stage in their life cycle, or vice-versa and the final classification of a form ultimately may involve the arbitrary designation of some stage in the cycle as the adult stage. The difficulty lies in that sexual maturity cannot always be used as a guide as in the case of the Metazoa. Form changes often are so pronounced as to lead the worker to designate as a new species an individual that may be merely a stage in the life cycle of an already well-known species, or, stages in the life-cycles of two species may be mixed. Such mistakes have been committed even by experienced workers.

The physiological vigor and activity of protozoa appears not to be constant throughout the life cycle. Without engaging in the controversy that has been waged around the proposition originally advanced by Weismann that protozoa are potential germ cells and, as such are potentially immortal, which has led to a discussion as to the exact significance of fertilization, it may be said that there is considerable evidence to indicate that protozoa exhibit phenomena of physiological old age which appear to be obliterated by process of fertilization or nuclear reorganization. Indeed, the rôle of fertilization in rejuvenescence appears to have been clearly shown by Calkins in his classical experiments on *Uroleptus mobilis*, a free living ciliate.

So far, little has been done to correlate such findings with the life cycles of the parasitic protozoa, but they suggest interesting clinical possibilities.

These, then, constitute the facts that, under Calkins' definition, are "collectively known as the life cycle."

Basis of Classification. In a work such as this it is neither necessary nor desirable to go deeply into the matter of classification. The four main groups will be defined with such of the sub-groups as contain species that are likely to be encountered by the worker in veterinary medicine. The organs of locomotion afford the best basis for the designation of the main groups but even that criterion has its limitations and inconsistencies. Under this system, the protozoa are grouped into the Sarcodina or amœboid forms; the Mastigophora or flagellated forms; the Sporozoa, which are parasitic forms reproducing usually, by spore formation, and the Infusoria or ciliated forms. Each of these groups contains species commonly occurring as parasites in domestic animals.

THE STRUCTURE OF THE PROTOZOA

Aside from all discussion as to the unicellular or the non-cellular nature of the Protozoa, the fact remains that they fall, in part, within the generally accepted definition of the cell as a mass of protoplasm differentiated into cytoplasm and nucleoplasm, or, better still, as Wilson[4] suggests, cytoplasm and karyoplasm. The difficulty lies in that the Protozoa pass beyond these limits. Like tissue cells they show a differentiation of their protoplasm into an outer or cortical layer known as the Ectoplasm from which arise the motile organs; and the inner or medullary area broadly referred to as the Endoplasm (cytoplasm), which contains the nuclear and digestive apparatus as well as other cell organelles characteristic of particular species. However, tissue cells of the Metazoa do not approach in complexity the structure of the more highly developed protozoa.

The general structure of the Protozoa may now be briefly discussed:

Adaptation to Parasitism. In dealing with the parasitic protozoa, it must be borne in mind that many species show very striking modifications and adaptations to their mode of life. Their habitat may lie in the lumen of the intestine in which event their general formation shows relatively little variation from that of the forms encountered in fresh or salt waters. On the other hand, a lumen dwelling form such as *Giardia* shows the acquisition of a suctorial disk for attachment to the mucous membrane, a

[4] Wilson, Edmund B., The Cell in Development and Heredity, 3e, 1925, New York, Macmillan.

most striking adaptation to a parasitic mode of life. Other species, as the Coccidia and *Leishmania*, pass trophic life in cells or tissues many of them having lost the power of locomotion in the process of adaptation, while others, such as *Entamœba histolytica* and *Balantidium coli* are able to roam about among the cellular structures of their hosts, gathering food and multiplying as they progress.

The Ectoplasm. Ectoplasmic structure is seen in its simplest form in the amœboid forms. Here the ectoplasmic differentiation in normal individuals may be so slight as to be barely visible when the organism is at rest. There always is a tendency for protoplasm to condense or stiffen under outside influences and this sometimes leads to the formation of fine membrane-like pellicles. For the most part, the ectoplasm of the amœbæ appears as a clear, hyaline cortical area that tends to become more prominent as the organism approaches death, which is one of the numerous reasons why protozoa should be studied in conditions as closely approximating the normal as possible. They are delicate creatures, extremely sensitive to untoward environmental conditions. Much endeavor has been expended in the past in depicting the pathology of the protozoa under the mistaken idea that the normal animal was being studied.

The flagellates, however, show very definite ectoplasmic modifications. All of them, of course, have one or more flagella and most of them have very definite cell membranes or pellicles. Notwithstanding this, however, their bodies are very plastic and frequently exhibit marked changes of form which, in some cases, progress along the body in a manner that strikingly resembles the waves of peristalsis in the intestine. These are styled euglenoid or metabolic movements. Somewhat similar changes of form are seen in the trichomonad forms as they approach death. They frequently are incorrectly spoken of as pseudopodial formation. Comparison of these movements with those of an active amœba will quickly show where the difference lies.

Another ectoplasmic modification encountered in the parasitic flagellates is the undulating membrane which is seen most strikingly in forms such as *Trypanosoma* and *Trichomonas*. The undulating membrane of the flagellates is an extension of the ectoplasm and always is bordered on its free edge by a flagellum which runs a more or less longitudinal, spiral course down the body, frequently terminating as a free lash. Careful staining of this structure often will demonstrate a longitudinal series of fibrils which have been interpreted by some workers as myonemes or primitive muscle fibres. As has been said, these ectoplasmic motor modifications are in definite relation to the nucleus and other kinetic structures.

Ectoplasmic development in the Protozoa reaches its height in the ciliates. The distribution of the cilia is subject to considerable modification. In some forms the entire body is invested with a dense coat of cilia; in others the cilia are sparse while in still others they may be restricted to certain parts of the body. As in the case of the flagella of the Mastigophora, cilia may be employed in food taking as well as in locomotion. Moreover, cilia may be bunched to form brush-like organelles known as cirri, or they may fuse side to side in short blocks—membranelles; or long sheets—membranes. It will be seen that the undulating membrane of the ciliates is distinctly different from that of the flagellates in that it is formed by the side to side fusion of cilia, whereas in the flagellates it is a sheet-like prolongation of the ectoplasm. In all cases, whether flagella or cilia or modifications of the latter, they are in relation to kinetic bodies—basal granules—which, in the flagellates, may function as centrosomes and become involved in nuclear division.

In many species of protozoa the rôle of the motile organs in food gathering is equally important. As amœbæ progress by the throwing out of pseudopodia, so do these same pseudopodia seek out and engulf objects that lie in their path. Many flagellates among the parasitic species, such as *Trichomonas* and *Chilomastix* have definite mouth openings or cytostomes which, while they, of course, originate in the ectoplasm end blindly in the endoplasm. Contractile or excretory vacuoles do not as a rule occur in parasitic species, conditions of osmotic pressure rendering them unnecessary. They are seen in *Balantidium*, however, and are always to be regarded as ectoplasmic organelles. Certain more highly organized species have a definite anal excretory pore or CYTOPYGE.

It must be borne in mind that ectoplasm and endoplasm cannot always be considered as two sharply differentiated topographical regions of the protozoan body. In the amœbæ, when the animal is active, its protoplasm is in a constant state of flux, and endoplasm and ectoplasm are interchangeable. That is to say, when the organism is forming pseudopodia what is endoplasm one moment may be ectoplasm the next. The ectoplasm of the flagellates may be said to consist of an extremely thin membrane or periplast which may contain contractile elements. Such is the case with the Sporozoa. The ectoplasm and its organelles, however, often reach a high degree of complexity in the ciliates and may be the most conspicuous feature of the structure.

The Endoplasm. Adaptations and modifications to a parasitic mode of life have tended to simplify the endoplasmic structure of most of the parasitic protozoa. Many of them simply absorb their food, which is in a

soluble condition, through the body wall and hence possess no digestive vacuoles. As a result many of the flagellates, and the Sporozoa as a group, exhibit no fixed endoplasmic structures aside from those of nuclear and kinetic origin. However, various bodies of metaplastic nature concerned in the metabolic processes are seen in all species and sometimes play their part in the creation of confusing cytological pictures. Fat globules, glycogen, the enigmatical "chromatoid bodies" seen in the cysts of parasitic amœbæ, pigments such as the hæmozoin of the Hæmosporidia and reserve food material of varying nature stored in preparation for periods of activity in reproduction are numbered among the cell inclusions that are encountered in different species. These are entirely apart from cell organelles, but they often impart a very distinctive appearance to the organism.

Species that ingest solid food such as many of the lumen-dwelling amœbæ, flagellates and ciliates form digestive vacuoles in the endoplasm. They always are conspicuous and are readily identified. In the amœbæ they are formed irregularly and in harmony with the movements of the pseudopodia, but in the flagellates and ciliates they form at the base of the cytostome or mouth and then are carried about in the cytoplasm.

The general appearance of the endoplasm varies with the different species and is more or less related to their mode of nutrition. In many the endoplasm is finely granular and homogeneous. In others, the presence of metaplastic substances gives it a more coarsely granulated appearance. Still others exhibit an alveolar type of endoplasm, the alveoli being fine and circular in outline. A notable exception to this is seen in the lumen-dwelling amœba of man *Entamœba coli* in which some of the protoplasmic alveoli are elongated or cleft-like in appearance.

The student of the protozoa must, however, be on his guard in the interpretation of vacuoles. Large, bubble-like vacuoles occupying relatively large areas or leading to distortion of the outline of the organism are, as a general rule to be regarded as evidence of degeneration and imminent death of the animal.

The Nucleus. Discussion of cell organelles that are characteristic of the free-living species of Protozoa, only, is purposely omitted here and the same policy will be followed in the description of nuclei and other structures that are in relation to the kinetic activities of the organisms. The nucleus will be discussed mainly from the viewpoint of its morphology rather than on the basis of its chemical constitution.

In the study of nuclei it is well for the beginner to carry three facts well in mind. These are:

1. In cell degeneration the first evidences of degeneration usually are seen in the nucleus.

2. There is no specific stain for chromatin. That is to say, there is no staining reagent that will stain chromatin and nothing else in the cell. As a matter of fact there are numbers of substances encountered in the protozoan cell that not only take up the nuclear stains, but in some instances stain more intensely than chromatin. The intensity with which chromatin takes up the so-called nuclear stains is not uniform at all periods in the life history of the cell. The reaction usually is more intense at periods of division or fertilization.

3. The best means available for the determination of the nature of bodies suspected to be chromatin or nuclei, is to study them in relation to the physiological processes in the cell. A body of true nuclear nature will sooner or later exhibit its rôle in karyokinesis or fertilization. This is a crude generalization, but it is impossible here to discuss the subject *in extenso*. Much misconception will be avoided if the observer will view with balanced skepticism any phenomena of apparent amitotic nuclear division. Definite mitotic spindles are formed in the dividing nuclei of the common species of parasitic protozoa. In binary or multiple fission, partition of certain cell organelles may take place by a process simulating amitosis which has led to their identification as bodies of nuclear nature. This will be considered further in the discussion of the "kinetonucleus" and "parabasal bodies" of certain of the parasitic flagellates. Moreover, in the dimorphic nuclei of the ciliates, while division of the micronucleus is accompanied by the formation of a definite spindle figure, the division of the macronucleus (meganucleus) seems to be a simple partition of its body accomplished coincidentally with division of the cell body as a whole, no mitotic figure being visible. Nuclear division in Hæmosporidia, such as *Plasmodium* and *Piroplasma*, as seen in most preparations, seemingly is amitotic, but, it must be recalled that the cytological methods employed in the study of these forms are barbarous in the extreme. Drying and subsequent fixation by heat or alcohol suffice to obliterate or distort delicate cellular structures, and stains of the Romanowsky type are not suited to studies of the nucleus. So it may be said that no accurate conclusions can be drawn concerning the structure or behavior of a nucleus that has been treated in that manner.

It is impossible to give a general description that will cover all protozoan nuclei for they vary greatly in form and, in some instances, in function as well. The most frequently encountered type of nucleus of the common species of parasitic protozoa is that which often is termed the "ring" or

karyosome nucleus. It is a nucleus of the vesicular type but in some instances the central mass of chromatin is so large in proportion to the nucleus as a whole that the impression given is that of a pyknotic nucleus. Indeed, it is only by the most careful staining and extraction that the structure of nuclei is demonstrated.

The karyosome type of nucleus usually is separated from the cytoplasm by a nuclear membrane which may be very difficult of demonstration in some cases and perfectly distinct in others. The framework of the nucleus, like that of the nucleus of an epithelial cell, is an achromatinic reticulum of *linin*. The inner aspect of the nuclear membrane is lined with a single layer of chromatin granules. In some instances these granules are relatively coarse and few in number; in others, as in the case of *Entamœba coli* as shown in Figure 63 they are finer and more numerous. This, usually, is spoken of as the peripheral chromatin. Occupying a central or sub-central position is the karyosome. This is composed of chromatin on a plastin matrix. While, in some instances there appears to be a body of centrosomic nature (the centriole), included in the karyosome, in other cases no such body can be demonstrated. The karyosome, therefore, is not to be regarded as a body of centrosomic nature. Neither is it a nucleolus. The nuclear alveoli of which the *linin* network probably is the optical expression contains the nuclear sap or enchylema. In some instances the area between the karyosome and the peripheral chromatin is almost, if not wholly devoid of chromatinic granules; in others there may be a so-called "nuclear cloud"—an aggregation of small to irregularly sized granules. Plasmosomes or true nucleoli are not likely to be encountered by the casual observer of protozoa. Plastin elements (those showing little affinity for chromatin stains) occur most conspicuously as the *linin* network and other supporting structures, and in nuclei such as are seen in the amœba *Iodamœba butschlii*. Such are the general features of the karyosomic type of nucleus.

It is necessary briefly to discuss the structure of nuclei occurring in the ciliates, because they present certain difficulties to the novice, both as to their structure, and as to their rôle in cell physiology.

As has been stated, one of the distinguishing characters of the Infusoria (ciliates) is the possession of two nuclei known respectively as the macro- or meganucleus, and the micronucleus. The former appears to be concerned in the vegetative processes as metabolism, while the latter is active solely in reproduction and allied processes. In *Balantidium*, the form most likely to be encountered by the veterinarian, the macronucleus is virtually a sack containing chromatin granules of varying size. These

granules are closely packed, rather irregular in size, but generally quite small. In some instances, however, they may reach a size of 1 to 2μ in their greatest dimension. The nuclear membrane is quite distinct. The nucleus as a whole is elongate. Usually, it is bent to kidney or even horseshoe shape. In other instances it may be straight and rodlike. Occasionally the diameter is increased at each end, giving it the appearance of a dumbbell. Usually it is surrounded by a relatively clear area of endoplasm. Roughly speaking, its length is approximately one-third to one-half that of the entire organism. It takes the nuclear stains intensely and, stained, is a most conspicuous object in the animal.

On the other hand, the micronucleus is a rather inconspicuous body and may escape casual inspection. In its vegetative state it is subspherical and does not exceed 5μ at its greatest diameter. Its flattened side is in close apposition to the nuclear membrane of the macronucleus, frequently lying there in a depression in the macronucleus, where its detection is exceedingly difficult. It has a nuclear membrane. The chromatin appears homogeneous, but it probably is granular or fibrillar in arrangement. Division of the macronucleus is direct and secondary to the division of the micronucleus which is mitotic in character.

The "Binucleata" and the Parabasal Body. Certain of the parasitic flagellates, including the trypanosomes and allied forms, were at one time considered to have two nuclei named respectively the trophonucleus and the kinetonucleus. This led Hartmann and his school to include a number of parasitic protozoa occurring among the Mastigophora and Sporozoa in a group called by them the "Binucleata." It was a heterogeneous collection of organisms showing no real relationships aside from bodies of fancied nuclear nature. The "Binucleata" survived to trouble investigators for many years until 1916 when Swezy[5] critically reviewed the evidence covering the organisms involved and reached the conclusion that the kinetonucleus is not a nucleus and probably, in no instance, arises from a nucleus. Her view is that "It is a structure correlated with an endoparasitic mode of life, and is part of the extranuclear motor apparatus. . . . . " It is regarded by her as a "kinetic reservoir." She has adopted Janicki's term "parabasal body" as a substitute for "kinetonucleus" and homologizes it with the chromatinic basal rod of *Trichomonas* also spoken of as the parabasal body. The "Trophonucleus" of *Trypanosoma*, therefore, becomes the nucleus. Even were the "kinetonucleus" a nucleus, it would be incorrect to call it a "micronucleus" as has been done by many writers—particularly among the French.

[5] Swezy, Olive, Univ. Calif. Pub. in Zoöl., xvi, (1916), no. 15, pp. 185–240

The Neuromotor Apparatus. This leads to a discussion of certain accessory organelles occurring in the parasitic flagellates and ciliates that constitute what is spoken of as the "neuro-motor apparatus." A full account of this would lead the reader far afield into the technical side of protozoology which would be out of place here. Suffice it to say that they constitute a complex of kinetic organelles that include flagella, cilia, basal granules and blepharoplasts in relation thereto, and parabasal bodies of various types which latter Swezy has interpreted as kinetic reservoirs. The axostyle of *Trichomonas* and other forms also is included. These are held to be connected with each other and with the cell nucleus by a series of fibrils that appear to be of a conducting nature. Minchin[6] has defined the blepharoplast as "A centrosome which is in relation to a motor cell-organ." In short, it is an organelle that may exhibit two definite functions—(1) a kinetic body or basal granule in relation to a flagellum, and (2) a centrosome functioning in division. The term blepharoplast is applied to this granule-complex, but in some instances it happens that the blepharoplast becomes separated into two granules one of which is in distinct relation to the motor organ and the other becoming active only when the cell is dividing.

The tyro will do well to refrain from too liberal interpretations of chromatin masses as "chromosomes." Chromosomes have been demonstrated during nuclear division in some species, but similar interpretations made in connection with other species are open to grave doubt and, so far, lack confirmation. As a means for the identification of species, chromosome counts cannot be said to have as yet established themselves as criteria in the Protozoa.

Sexual Phenomena. A few words of caution also must be uttered concerning the sexual processes—termed SYNGAMY—for many workers have been led astray. In some cases, sexually differentiated gametes or sex cells are produced; in other instances the process takes place between two individuals indistinguishable from the ordinary vegetative cells. There are two general lines along which the performance takes place. In the first we have copulation or, as it is sometimes styled, total karyogamy in which the two individuals fuse completely and never regain their individuality. In the second, known as conjugation or partial karyogamy, the union of the two cells is temporary, persisting only long enough to allow for an interchange of nuclear material. In free-swimming types the union usually is side to side along the long axis of the cell. In this way

[6] Minchin, E. A., An Introduction to the Study of the Protozoa, with Special Reference to the Parasitic Forms, (1912), London, Edward Arnold.

it often has happened that cells have been interpreted as "conjugants" or "in copulation" when, as a matter of fact they simply were undergoing division, or had fortuitously come in contact with each other. It will help the beginner if he will remember that division in the flagellates is parallel to the long axis of the cell while in the ciliates it is transverse. This often will give him pause and lead him to analyze the state of the nucleus which affords the real basis for the interpretation of processes in syngamy. Moreover, it sometimes happens that amœboid cells meet and fuse without the interchange of nuclear material. Such a process is spoken of as plastogamy or plasmogamy. It has no sexual significance. Temporary fusions or agglomerations take place under peculiar conditions as, for instance, agglomerations of trypanosomes, and must be interpreted on other bases. In the end, all these things stand or fall on the state of activity of the nucleus—not on the general form that the organism may exhibit at a particular time.

Much trouble and misconception will be saved if the worker will bear well in mind that the protozoa are exceedingly delicate organisms whose structure is greatly modified by conditions that only slightly depart from those under which the animal normally lives and reproduces. In consequence, an immense amount of ink and paper has been used to depict the normal cytology of the organism, the picture presented unhappily being that of the pathological cytology. There is a vast difference between the hastily fixed and stained preparation made for the identification of a species already well known, and the skill and care required to present the details of one that is under investigation. Not only must the animal be killed and its protoplasm fixed before degeneration has started on its destructive work, but the best reagents and methods of the cytologist must be employed. The beginner will do well to acquaint himself with some of the standard cytological methods as set forth in Lee's Vade Mecum[7] or some work of that type, before undertaking to study and describe an organism with which he is not familiar.

For purposes of identification of the ordinary tissue-dwelling forms as coccidia and amœbæ the ordinary methods of the histo-pathologist will suffice provided the organisms are promptly and properly fixed. Ordinary blood methods will suffice for the identification of hæmosporidia and the hæmoflagellates, but the difficulties of applying more precise methods to the study of these organisms undoubtedly has prevented the acquisition of much important knowledge bearing on their structure and life history.

[7] Lee, Arthur Bolles, Microtomists' Vade Mecum, 8e (edited by J. Bronte Gatenby), London, J. and A. Churchill.

Before proceeding to an account of a few of the more important species a few words must be said about methods of nutrition.

THE PHYSIOLOGY OF THE PROTOZOA

Nutrition. The parasitic protozoa exhibit two types of nutrition—the saprozoic (saprophytic) and holozoic. Saprozoic forms are those that have no mouths and which absorb their food in a fluid state through the body wall. In some instances this food may be predigested to a greater or lesser degree; in other cases the organisms are able to construct their own proteins from simpler substances so absorbed.

Other forms capture, devour and digest their food, which consists of ready made proteins. These are broken down and resynthesized into protoplasm by processes essentially similar to those of the higher animals. This is spoken of as the holozoic type of nutrition.

Mode of Life of the Parasitic Forms. Parasites are, of course, entirely dependent upon what they can pick up in the line of food in or on their particular host. Usually, they show a highly specialized adaptation to a uniform diet and do not readily depart therefrom. The writer has found it exceedingly helpful to students in considering the parasites of the intestinal tract to indicate that certain of them are essentially vegetarians while others are carnivorous. The lesson from this is that species which clearly subsist on bacteria and other lowly plant forms may not, in the general run of instances, be regarded as likely to attack the tissues; while those that do not take in vegetable forms, but that can be shown to devour cells or their products are exceedingly likely to live in the tissues and produce a greater or lesser degree of injury to them. However, it frequently occurs that protozoa take in nutritious substances to which they are not adapted, or even non-nutritious or inert substances. Such substances may be carried about in the cytoplasm for a while, but eventually they are disgorged. The criterion on which judgment should be based in such instances is not that the organism has ingested such and such a substance, but should lie in the answer to the question as to whether it has *digested* the substance. Were this rule always followed several supposedly pathogenic species would be shorn of their terrors.

Tissue Reactions. As has been said, the phenomena in the host attendant upon protozoal infection are different from those produced by bacteria. A definite inflammatory reaction in tissue infested with protozoa, always should awaken suspicion of concomitant bacterial infection. The same may be said with regard to toxæmia. In general, it may be said that protozoa do not produce diffusible toxins. This is known to be the case

with the amœbæ, the coccidia, many of the flagellates, and the ciliates. Phenomena attendant upon infection with trypanosomes, sarcosporidia, and the malaria parasite require other consideration. An uncomplicated protozoal infection of the tissues usually leaves the road clear for prompt repair by the usual processes. The method of attack upon the tissue is a non-toxic process of proteolysis, and it often happens that destruction of tissue and repair of the damage proceed more or less synchronously so that a given parasite may abide with its host over a considerable period of time, securing an abundance of food during its sojourn but at no time producing lesions that seriously incommode the host. Relatively seldom does it happen that a protozoal infection is rapidly fatal. There is lacking the explosive acuteness to these infections such as one sees in diphtheria, cholera or plague.

Commensalism and Parasitism. The whole trend in protozoal infection is toward the establishment of physiological equilibrium between the parasite and its host. Two grades of association are seen: (1) COMMENSALISM which for instance, is the situation existing in the case of the lumen-dwelling species of the intestine. Here, the parasite nourishes itself on substances that are not part of the body of its host—bacteria, food detritus and so forth. No demands are made upon the body substance of the host and, accordingly no pathogenic properties are to be ascribed to the parasite, and (2) PARASITISM (*sensu strictu*) in which the body substance of the host forms the food supply of the parasite. Even in the latter event the parasite is not of necessity lethal. Having in mind what has been said elsewhere in this chapter about a seeming transition stage toward tissue parasitism in certain trichomonad flagellates, it may be broadly stated that the two modes of life are not interchangeable. One always should inquire into the accuracy of one's observations upon a parasite that appears to be a commensal at one stage of its life cycle and a tissue parasite at another.

In reflecting upon these things it must be recalled that an existing protozoal infection represents the realization of untold years spent by a parasite in a struggle to secure to itself peace and security in the possession of a desirable source of food. Having attained that end the failure of that source of food supply through the death of the host, certainly does not redound to the advantage of the parasite in the general run of instances. On the other hand such a contingency may be a real calamity to the organism.

Such is the state of affairs during the trophic life of the parasite. During this period the parasite is feeding actively and its multiplicative rate is

high as the infection becomes thoroughly established. Eventually, comes the time when a new host must be sought, and then the old host ceases to be of importance to the parasite which has entered upon its propagative phase in the life cycle.

Intermediary Hosts and Transmission. Transmission of parasitic protozoa to new hosts, as in the case of the transmission of other parasites, is accomplished at great numerical loss which, of course, finds compensation in the production of enormous numbers of individuals in the propagative stage. It is impossible here to discuss all the methods by which protozoa make their way from host to host. The most common methods only can be barely outlined.

In the case of parasites of the intestinal tract, the organism ordinarily encysts. That is to say, it becomes invested with a more or less resistant capsule which enables the contained protoplasmic mass to survive conditions of moderate drying and other milder adversities of the outside environment. In some cases, to be sure, these cysts are very tough and impervious to unfavorable influences and also resist a high degree of desiccation. In other instances, as in the case of *Trypanosoma equiperdum*, the cause of dourine in horses, transmission is effected by direct body contact of the two hosts. A large group of protozoan parasites are transmitted by arthropod vectors. In certain species the parasites undergo a developmental stage in the invertebrate host and become infective to a new vertebrate host only on the completion of that phase. In other instances the arthropod may merely serve as a mechanical carrier through contamination of its mouth parts by parasitic organisms taken up from the blood stream of the first host and regurgitated into the body of the second host at the succeeding meal. In this case the arthropod virtually is an animated hypodermic syringe; it is not to be regarded as an intermediary host in the strict sense. Alternation of generations accompanies alternation of hosts, different developmental cycles occurring in the definitive and intermediary hosts.

One potential source of error occasionally is encountered in the study of the fæces. Encysted protozoa, such as coccidia, will be found in such numbers as to lead to the assumption that they represent a species parasitic in this host. However, investigation of the intestinal tract fails to reveal any tissue-dwelling forms. Further investigation leads to the discovery that the cysts originate in the tissues of another animal that has been eaten by the animal under study. The writer recalls an instance in which he discovered coccidial cysts in the fæces of a number of lizards in Manila. As Philippine lizards frequently are parasitized by coccidia,

and as the cysts in question were of a type he had not before seen, he investigated further. No intracellular forms were found, but fully developed cysts were found in the stomach together with masses of skeletal debris from various arthropods. This effectively disposed of the coccidium as a parasite of the lizard. It probably was specific to some arthropod forming the diet of the lizard. In this instance the lizard played an important rôle in disseminating this parasite, but was in no sense a true intermediary host.

Many other complex situations may arise in the transmission of parasitic protozoa from host to host, but it is impossible to discuss them here. For instance in some species (*i.e.*, *Babesia* and *Nosema*), the parasite may penetrate the ovary and be conveyed to the next generation in the developing ovum. There may even be two methods of transmission in order that the parasite may be protected during adverse climatic changes.

The attainment of the perfect state of parasitism—which involves harmonious relations between host and parasite to an ultimate degree—is the summation of a marvelous series of modifications and adaptations. These are most apparent in the parasite, but the host has played no small part. Many instances of a high degree of mutual adaptation are afforded by the parasitic protozoa and the student must ponder them carefully before deciding out-of-hand that a protozoön he has encountered—even in the tissues—is, *per se*, a lethal parasite.

Cultivation of Parasitic Protozoa. Allied to the nutrition of the Protozoa are the problems of cultivation. The main problem is much more simply expressed than met. It lies in the discovery of the appropriate food, and its administration under the appropriate physical and chemical conditions. The cultivation of free-living protozoa usually does not offer great difficulties. The difficulties are much greater with the parasitic protozoa, and have been overcome only in a relatively small number of species. The first notable success in the cultivation of the parasitic protozoa came in 1903 when Novy and MacNeal[8] succeeded in cultivating *Trypanosoma lewisi*. Their method, with slight modifications has since been applied to the cultivation of other trypanosomes and *Leishmania*. Another notable step was made when Bass[9] announced his success in cultivating *Plasmodium*, the causative organism of Malaria which method also has been extended to the cultivation of other hæmosporidia. The most recent con-

[8] Novy and MacNeal, Contributions to Medical Research, dedicated to V. C. Vaughan, Ann Arbor, Mich., (1903), 549.

[9] Bass, C. C., Jour. Amer. Med. Assoc., lvii, (1911), 1534–1535.

tribution of note along these lines has been that of Boeck and Drbohlav[10] who have succeeded in cultivating *Entamœba histolytica*, the dysentery amœba of man. This method has been extended to the cultivation of other intestinal protozoa of man. Strangely enough, however, and this illustrates the fastidiousness with which Protozoa choose their food, it not infrequently happens that a medium upon which one species will grow luxuriantly utterly fails to support another species within the same genus. Obligatory tissue parasites seem to offer the greatest difficulties in cultivation, the reasons being fairly obvious. Real advance in the cultivation of parasitic protozoa was not made until the methods of the bacteriologist were adopted. Certain forms present difficulties in that they live side by side with bacteria that grow in the same medium and make the obtaining of pure cultures a task of great difficulty.

Symbiosis is a term that frequently is misused in discussing the parasitic protozoa. One encounters statements to the effect that a certain protozoön has been cultivated "in symbiosis," with a bacterium, it also being clearly shown that the bacteria were devoured by the protozoön. Correctly used, the term symbiosis connotes a relation between two organisms living together by which each receives some essential substance from the other. At the same time, neither organism works harm to the other. The relation is one of mutual benefit, solely, the two organisms being largely dependent upon each other for existence. In the example cited the bacteria, far from receiving benefit from the protozoön, die that the latter may live.

We shall now proceed to a brief treatment of representative members of the four main groups:

THE SARCODINA

These are protozoa having motile organs in the form of changeable protoplasmic processes known as pseudopodia. These may be either simple or supported by a central rod of stiffened protoplasm, the axial filament. They range in form from those that are little more than minute masses of protoplasm to forms having elaborate shells, tests and skeletal structures. They have no affiliations with the bacteria.

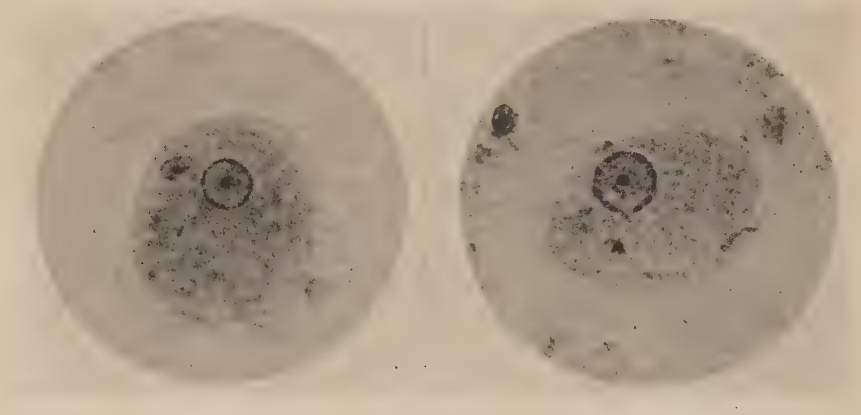
The Rhizopoda are the only Class in this sub-phylum containing species of medical interest. They are Sarcodina that form pseudopodia lacking an axial filament. In the Rhizopoda are included the many free-living and parasitic amœbæ.

Parasitic Amœbæ. As an example of this group we shall consider the

[10] Boeck and Drbohlav, Amer. Jour. Hyg., v, (1925), 371–407.

common intestinal parasite of man—*Entamœba coli.* This organism lives in the lumen of the large intestine probably occurring in greatest numbers in the cæcum. It lives on bacteria and other primitive plant forms, never invades the tissues and is not to be regarded as pathogenic in any sense of the word. In many ways it resembles *Entamœba histolytica*, the dysentery amœba, but at the same time it presents differences in structure that make it easy to distinguish between the two provided the material is fresh.

Like the dysentery amœba, *Entamœba coli* may be found in the fæces in any one of three stages in its life cycle: the active or trophic stage, the precystic stage or the encysted stage.



(a) (b)

FIG. 63. ENTAMŒBA COLI

Two individuals from the same preparation. The nucleus of (*a*) is almost normal in appearance; that of (*b*) shows marked degeneration. (Photomicrograph by Kelser from preparation by Professor Haughwout.)

Entamœba coli is a large, sluggish amœba. Rounded out individuals may measure anywhere from 18 to 40μ in diameter. The differentiation of ecto- and endoplasm is not especially marked. The endoplasm is granular and carries numerous food vacuoles that contain bacteria and other substances engulfed by the animal as it forages about. In addition to these the protoplasm often shows a vacuolated structure of a peculiar type. These vacuoles appear like clefts or, to quote Dobell, "dilated cracks with pointed ends." The nucleus, as will be seen from the photograph, is of the karyosome type. The karyosome is conspicuous, usually eccentrically placed in the nucleus, and there is a definite cortical "halo" about it.

The life cycle of this organism is only partly known. The assumption is that it reproduces by a process of binary fission, but no detailed set of reliable observations on this has been made. It has been suggested by some workers that multiple division may occur, but no conclusive demonstration has been made of this, either. As the large trophic amœbæ are carried down the intestine by the movement of the fæces, they prepare for encystment to insure their passage to another host. The animal stops feeding and the daughter amœbæ, formed by the division of large forms, do not grow to full size, but round out and lose most of their activity. Their endoplasm becomes clear of food inclusions, and eventually a cyst forms around the organism. Three successive nuclear divisions occur so that the mature cyst contains eight small nuclei of the karyosome type. A huge vacuole containing glycogen forms in the early stages of this division. It usually has completely disappeared by the time the cyst has reached the 8-nucleated stage. Dark staining, irregularly shaped bodies also are found in some cysts. These are called chromatoidal bodies. Their function is not clear, but they appear to represent a reserve of some kind that is utilized by the organism during its passage from host to host, or from host to oblivion after encystment. These cysts open in the intestine of a new host, and eight small amœbæ become free and develop to the adult trophic stage. Details of this have not been worked out.

The group of intestinal amœbæ is one of great interest. Those infesting man and some animals have been ably discussed by Dobell in his monograph.[11] They are found parasitic in a large number of animals, and five species are known to occur in man. Amœbæ found in monkeys, frogs and certain rodents are indistinguishable from corresponding forms that are found in man. With the exception of *Entamœba histolytica*, the dysentery amœba of man, these organisms seem to be harmless commensals living the lives of scavengers in the intestinal tract. However, from time to time, reports are made of "amœbic dysentery" in the dog and in other animals and there undoubtedly is opportunity for considerable interesting work on the pathology of intestinal protozoal infections in the lower animals.

THE MASTIGOPHORA

These are protozoa having motile organs in the form of one or more vibratile or undulating whip-like processes known as flagella. These have been shown, in many species, to be in relation, through a fibrillar

[11] Dobell, Clifford, The Amoebae Living in Man: A Zoölogical Monograph, (1919), London, John Bale, Sons and Danielsson, Ltd.

system, with nuclei and centrosomes, and other bodies of a supposed kinetic nature styled by Kofoid and his co-workers "the neuromotor system." These occur solely in the parasitic species. In certain parasitic forms the flagellum forms the border of a thin sheet of ectoplasm known as the undulating membrane.

Several of the sub-groups here are represented by species parasitic in lower animals. In the past, most authors have considered the Spirochætes as Mastigophora, but the tendency now, in view of their close affinity with the Cyanophaceæ, is to deliver them to the bacteriologist to whose methods they would obviously seem more suited. The Mastigophora include the immense group of Trypanosomes, the related genera such as *Herpetomonas*, *Crithidia*, and *Leishmania*, and a large group of intestinal parasites including *Trichomonas*, *Chilomastix* and *Giardia*.

The Trypanosomes. The trypanosomes form an enormous group of blood parasites of vertebrate hosts. They are closely related to the other flagellated forms *Herpetomonas* and *Crithidia* which are found in the intestinal tracts of various arthropods.

The body of the typical trypanosome is elongate and pointed at each extremity. The anterior or flagellar end usually is more sharply tapering than is the posterior or aflagellar end. However, many trypanosomes are distinctly polymorphic varying at different times through gradations of forms extending from those that are short and stumpy to those that are long and serpentine. The tendency is to regard these variations as growth stages.

The nucleus, which when properly fixed and stained, is seen to be of the karyosome type, lies near the center of the body. The parabasal body ("kinetonucleus") usually is found behind it, but there are exceptions to this. Sometimes the two lie closely together, but usually they are separated by a considerable interval. Lying near the parabasal body is the basal body or blepharoplast from which the flagellum arises. The flagellum penetrates to the surface of the body and then pursues a sinuous course toward the anterior extremity. Gaining the surface of the body it forms the outer margin of the undulating membrane. This outer margin is considerably longer than the body of the trypanosome, as a result of which the undulating membrane is thrown into folds. The flagellum in many forms is continued beyond the extremity of the organism as a free lash; in others it may terminate abruptly at the extremity. The peristaltic movements of this undulating membrane are reversible so that the animal is able to move either forward or backward.

The general appearance of the cytoplasm of trypanosomes varies. In

some species it appears to be finely and in others more coarsely granular. Frequently the cytoplasm contains numerous granules that stain deeply with hæmatoxylin and other nuclear dyes. They, probably, are metaplastic.

(a) (b) (c)

FIG. 64. TRYPANOSOME UNDERGOING BINARY FISSION (KELSER)

a, parabasal body dividing; *b*, nucleus has completely divided; *c*, trypanosome splitting to form two organisms.

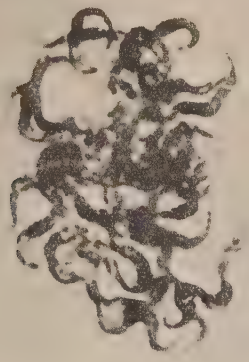


FIG. 65. AGGLOMERATED TRYPANOSOMES (KELSER)

Transmission of trypanosomes infesting land animals appears to be accomplished in a large proportion of instances through arthropod vectors. *Trypanosoma equiperdum* already has been mentioned as an exception to

this generalization. In many species arthropod transmission appears to be direct; in others there is a developmental cycle within the arthropod. The complete life history remains to be worked out in the case of any trypanosome. So far as this has been accomplished, the parasite appears to lose its distinctive trypanosome characters in the insect host, and take on characters more closely resembling *Herpetomonas* and *Crithidia*.

As in the case of the other flagellates, reproduction in the trypanosomes takes the form of longitudinal binary fission. In some instances division of the cell body may not be completed, the animals remaining attached by their aflagellar ends. Successive divisions take place with the resulting formation of rosettes. It is the opinion of the writer that the characterization of this process as "schizogony" is erroneous. Care must be taken to avoid confusing this appearance with that seen when groups of trypanosomes agglomerate by their flagellar ends in reaction to some unfavorable influence. This latter phenomenon can be demonstrated by adding to a suspension of trypanosomes a little blood serum from an animal affected with trypanosomiasis. The products of division, especially when the division rate is high, are not always equal in size.

In some species it has been shown that definite phases in the life cycle occur in the internal organs of the vertebrate host as the lungs and the spleen. *Trypanosoma cruzi* and *Trypanosoma evansi* are examples of this.

Many trypanosomes appear to be quite innocuous to their hosts as witness *Trypanosoma lewisi* of the rat and *Trypanosoma americanum* of cattle. The same condition exists in many wild animals as was shown very early in the investigations into sleeping sickness in Africa. In such cases these hosts often serve as reservoirs for the infection of other animals to whom the parasites may be pathogenic. The reservoir condition probably is the expression of complete adaptation following the long association of the two animals, for trypanosomes introduced from such a source into new hosts are apt to produce marked pathogenic or even lethal effects in them, although a certain degree of tolerance appears to be rapidly acquired. This, however, may be lost by passage through another host and subsequent reinoculation into the partially tolerant host.

Tissue Invasion by the Trichomonads. Mention must be made, in passing, of the work of Hadley[12] on infections with *Trichomonas* in turkeys suffering from blackhead, in which he has presented evidence to show that these flagellates are capable of invading the tissues through the route

[12] Hadley, Philip B., Bull. 166, Agr. Ex. Station, Rhode Island State College, (1916); (See also: Bull. 168, *Ibid.*, and Jour. Med. Res., xxxvi, (1917), 79).

afforded by the goblet cells of the intestinal mucosa. Wenyon[13] also has reported the same occurrence in man on evidence that is fairly convincing Kessel[14] reports finding the organism in the pus of a liver abscess in man, and the writer[15] once received a quantity of pleural exudate removed from a man, in which he found trichomonads indistinguishable from those commonly occurring in the intestinal tract. It has long been known that intestinal flagellates of birds and reptiles occasionally invade the blood stream and may be found there in large numbers. However, very little is known about the circumstances under which these organisms invade extra-intestinal parts of the body or, indeed, what they accomplish after they get there. The writer[16] also has shown that certain strains of trichomonads of the intestinal tract of man may depart from a vegetarian diet and show evidence of a beginning adaptation to a carnivorous diet in that they are able to digest red blood corpuscles. It is his view that these strains are passing through a well-marked stage in evolution from commensalism to obligatory tissue or blood parasitism. Of necessity, however, this matter will have to be held *sub judice* for a considerable period of time. In the meanwhile it is unwise to ascribe pathogenic properties to any of the intestinal flagellates until the protozoölogical evidence can be pooled with the findings of the pathologist. Some of the evidence on these points was critically reviewed by the writer[17] in 1918 and much of it will not hold water.

THE SPOROZOA

The most stable characteristic of this group is that all species are parasitic. It really constitutes a catch-all for species that cannot be properly placed until their full life histories are known. Reproduction in nearly all species is by spore formation, but exceptions are found among the Gregarines where certain species reproduce by division or multiple budding. In a general sense the Sporozoa have no motile organs most of them leading a relatively sessile existence. However, Gregarines are able to execute a vermiform motion and in many instances gametes are flagellated. Some species show affinities with the flagellates and others with the amœboid forms.

The Sporozoa are divided into two main sub-groups—the Telosporidia in which the life of the individual ends with sexual spore formation, and the

[13] Wenyon, C. M., Jour. Trop. Med. and Hyg., xxiii, (1920), 125–126.
[14] Kessel, John F., Jour. Parasitol., xi, (1925), 151–152.
[15] Haughwout, F. G., and de Leon, W., Philippine Jour. Sci., xiv, (1919), 207–219.
[16] Idem, ibid.
[17] Haughwout, Frank G., Philippine Jour. Sci., Sect. B, xiii, (1918), 217–259.

Neosporidia in which spore formation does not end the life of the individual, but may occur during the active life of the parasite without interfering with the vegetative processes.

Among the Telosporidia are found the "Coccidia," a large group of cell parasites; the Hæmosporidia, or blood parasites, such as *Hæmogregarina*, *Plasmodium* and *Piroplasma*. Wenyon[18] recently has reviewed the literature on the Hæmogregarines and has pointed out several fallacies and misconceptions concerning them and other bodies occurring in the blood that have been interpreted as Hæmogregarines. Aside from its value as a review of this group, the paper points many valuable lessons bearing on the pitfalls of protozoölogy. It may be read with profit even by those who are not especially interested in the Hæmogregarines. The only genus of veterinary interest in the Neosporidia is *Sarcocystis*, the common muscle parasite.

The Coccidia. In many ways the most interesting forms of Sporozoa and those of which we have the most precise knowledge are the "Coccidia." They are widely distributed in a great variety of hosts both vertebrate and invertebrate. Our present knowledge of them is based largely on Schaudinn's classical description of *Eimeria* (*Coccidium*) *schubergi* of the centipede *Lithobius forficatus*, in which he worked out the details of the complex coccidian life-cycle. The literature on this group of parasites is enormous. The writer would direct attention to the paper by Wenyon[19] on the coccidia of cats and dogs. The paper includes bibliographic reference to many of the more important papers on coccidiosis. Those who desire to look into the pathology of coccidiosis are advised to read the paper of Tyzzer[20] on coccidiosis in rabbits.

Intestinal coccidia have been regarded as essential epithelial cell parasites, but it is now known that they may be found in the deeper structures of the villi although their occurrence there probably is fortuitous. Those of medical and veterinary interest fall in the genera *Eimeria* (*Coccidium* is a synonym of *Eimeria*), and *Isospora*. The distinctions between the two genera will be explained later. Although coccidia destroy their host cells and are, in that sense pathogenic, they are not, in all cases, to be regarded as lethal parasites. There are a few exceptions to this rule. Another point to be borne in mind is that coccidial infections tend to be self-limited. There is an active endogenous cycle within the host during which the parasites multiply at an enormous rate and the in-

[18] Wenyon, C. M., Trop. Dis. Bull., xx, (1923), 527.
[19] Wenyon, C. M., Ann. Trop. Med. and Parasitol., xvii, (1923), 231–288.
[20] Tyzzer, E. E., Jour. Med. Res., n.s., ii, (1902), 235.

fection may involve large areas. In time this multiplicative endogenous phase gives way to a propagative exogenous phase and the trophic forms develop into sexual gametes. Fertilization takes place followed by encystment of spores which pass out to infect new hosts. This process eventually involves the entire stock of trophic forms with the result that the host is completely purged of its infection. No immunity is conferred, however, and super- and reinfection frequently occur. The cysts of coccidia are extremely resistant and it is exceedingly difficult to disinfect ground that has become contaminated with coccidial spores.

The terminology involved in the study of the sporozoan life-cycle seemingly is involved, but the stages of the cycle develop in such beautifully logical sequence and are in such harmony with the underlying principles of metazoan embryology that the terminology quickly loses its terrors. There is little difference between it and the terminology employed in describing the life cycle of the malaria parasite.

The following outline of the life cycle of a coccidian must be held to apply to forms that undergo development in the epithelium. There are lacking several important details in the life cycle of *Isospora bigemina*, that, according to Wenyon, undergoes development in the submucosa of the intestinal villi in the cat. The reader is referred to Wenyon's paper for such details as have been worked out.

The life-cycle of a coccidium may be divided into two phases: (1) the trophic or multiplicative phase which takes place in the host, and which is unaccompanied by sexual processes, and (2) the sexual or propagative phase (Sporogony) which starts with fertilization between differentiated gametes in the host, but usually is completed outside. *Isospora bigemina* forms an exception to this rule in that the entire performance takes place within the host and fully developed cysts pass out in the fæces. It is the view of Wenyon that the cysts in this species only reach the lumen of the intestines when they are liberated through the breaking down of the tips of the villi. Accordingly, their appearance in the fæces is very irregular, and they may be missed altogether unless several examinations are made of the fæces. These two phases are sometimes spoken of as the endogenous and exogenous phases respectively. There is no essential difference between the endogenous cycles of *Eimeria* and *Isospora*. The distinction between the two genera lies in the formation of the spores.

Infection is contracted through the ingestion of cysts of the organism. These pass through the stomach, but open in the small intestine liberating the Sporozoites which are the infecting form of the parasite. The sporozoites are capable of making their way to the surface of the epithelium and

boring into the cells. Having entered they become Trophozoites and proceed to feed on the cell substance. They lie in vacuole-like spaces in the host cell and gradually grow until they practically fill the cell and have crowded the nucleus against the basement membrane. They then become Schizonts and undergo a process of multiple fission called Schizogony. The nucleus divides several times to form a number of daughter nuclei, eight, twelve or possibly more according to the species. Each nucleus becomes surrounded by a mass of cytoplasm and eventually a bundle of elongated cells is formed. These are called Merozoites. They closely resemble the sporozoites and like them, infect new cells after they have made their way into the lumen of the gut. Entering the cells the merozoites become trophozoites and this cycle is repeated many times, the infection becoming more and more extensive until finally a large area is involved.

Gradually, however, the merozoites instead of developing into trophozoites grow into new types of cells—the Gametocytes which are the progenitors of the germ cells or Gametes. Development then proceeds along two lines—one towards the production of Microgametes, flagellated cells resembling spermatozoa and corresponding to them in function, and the other towards the production of Macrogametes, inert cells resembling ova and virtually functioning as such.

Microgametes develop from the Microgametocytes by successive nuclear divisions in the latter until 2,000 or more of these tiny flagellated microgametes are formed. Ultimately these become free in the lumen of the gut, swim about and some of them find their way to macrogametes which they fertilize just as the spermatozoön fertilizes the ovum.

The Macrogametocytes, on the other hand enlarge and store up nutritive matter to be employed in the series of cell divisions following fertilization and to tide over cell-metabolism during the waiting period before establishment in a new host. Nuclear reduction analagous to maturation may occur. In some cases, in the Sporozoa, this is accomplished before, and, in other cases, after fertilization. With penetration of the microgamete into the macrogamete a fertilization spindle is formed between the two pronuclei and the macrogamete which, meanwhile, has invested itself with a membraneous covering becomes a Zygote, and, with hardening and thickening of the membraneous covering—an Oöcyst. In this stage it usually leaves the body of the host in the fæces. It is at this point that the distinction between *Eimeria* (*Coccidium*), and *Isospora* (*Diplospora*), becomes most evident.

If the zygote is one of *Eimeria*, two nuclear divisions occur with the resulting formation of four daughter nuclei. Each of these nuclei be-

comes the center of a protoplasmic mass—the SPOROBLAST. In the case of *Isospora* only one nuclear division takes place with the formation of two instead of four sporoblasts. This constitutes the prime difference between the two genera.

Next, each sporoblast secretes a membraneous covering which, with the contained sporoblast, becomes the SPOROCYST. In this manner, the protoplasmic masses are protected by two coverings and they are, accordingly, exceedingly resistant to untoward environmental conditions. In the case of *Eimeria* there is cleavage of the protoplasmic mass in each sporocyst into two falciform cells which occupy the sporocyst with a small mass of unused or residual protoplasm. These falciform bodies are the SPOROZOITES. In the case of *Isospora* four sporozoites are formed in each sporocyst so that while the members of each genus produce eight sporozoites in all, in Sporogony, these are apportioned as two in each sporocyst in the case of *Eimeria* and four in *Isospora*. This completes development of the cysts which now are infective to the appropriate host, bringing us back to the point from which we started.

The PIROPLASMATA are Hæmosporidia parasitizing and destroying the erythrocytes of their hosts, the liberated haemoglobin being excreted by the kidneys giving rise to a hæmoglobinuria, the condition being popularly styled "red-water." The organism is transmitted from host to host by ticks in whose bodies a developmental stage occurs. The vertebrate hosts include cattle, sheep, horses, and dogs. Systematically, the Piroplasmata form a provisional group, the affinities of which cannot be settled until complete life histories have been worked out. Among the better known members of the group, as it stands at present, are:

Babesia spp. Parasitic in horses, oxen, sheep and dogs.

Theileria spp. The cause of East Coast cattle fever in Africa.

There are a number of other genera included in this group. The geographical distribution is wide, piroplasmosis having been reported from North and South America, Europe, Africa, Australia, the Philippine Islands, India and other parts of Asia.

In some respects the intracorpuscular stage in the cycle of this parasite recalls that of the malaria parasite. In the genus *Babesia* they are round, pyriform or irregular (amoeboid). In *Theileria* they may be rod-like or round and smaller than those of the other genus. The nucleus in *Babesia* is described as of the karyosome type and the cell contains dark-staining granules that have been interpreted as "chromidia." The organism grows well on Bass's glucose-blood medium.

The pear-shaped forms of *Babesia* penetrate the host corpuscles. They

round out and grow at the expense of the substance of the corpuscle. At the limit of growth, and just before division, the parasite becomes amoeboid and the chromatin of the nucleus condenses into a pyknotic mass. This mass gives off a bud which forks and divides into two buds which migrate towards the surface of the body enveloped by separate processes of cytoplasm. These two nucleated, bud-like processes grow, the whole parasite at this stage forming the figure of a "Y" until eventually the entire parent mass has been resolved into two pear-shaped bodies joined at their pointed ends. The two daughter cells then separate, leave the now depleted corpuscle and penetrate new corpuscles.

In the body of the tick, the parasites are received in the stomach where the pear-shaped forms, alone, survive. These undergo developmental changes which some writers believe lead to the formation of gametes and ultimately zygotes. From these a form develops which is believed to be analogous to the oökinete stage of the malaria parasite. These pass through the gut and into the ovary, coming to rest in the ova. Later, they may be found in the cells of the developing embryos of the tick. In the end the infective forms of the parasite reach the salivary glands in the tick and are inoculated into the vertebrate host at the next feeding.

It is the belief of Brumpt[21] who has critically reviewed the subject of piroplasmosis in cattle, that there are seven species of piroplasms. He also recognizes two species of *Anaplasma*. He differentiates between these on biological as well as pathological bases. All these species (with the exception of *Theileria parva*) are held to be inoculable into uninfected animals. He regards a specific reinfection as impossible for the parasites of the original infection persist in the blood for years after clinical recovery. Here, again, he excepts *Theileria parva*. His contention is that the test of natural immunity affords a standard of specific validity. That is to say, if an animal that has been clinically cured of a piroplasmosis of one kind or another reacts to the bite of a tick that is known to be infected with a pure strain of piroplasm, the last infection is specifically distinct from the first. He also calls attention to the value of a study of the physical reactions of the host in connection with specific determinations.

Brumpt[22] in discussing the transmission of canine piroplasmosis in Tunisia by *Rhipicephalus sanguineus* states that the progeny of infected females transmit not as larvæ or nymphs but only as adults, and that

[21] Brumpt, E., Bull. Soc. Path. Exot., xiii, (1920), 416–460.
[22] Brumpt, E., Bull. Soc. Path. Exot., xii, (1919), 757–764.

nymphs fed on infective blood are not infective as adults. He calls attention to the contrasts between bovine piroplasmosis where the progeny of infected mother ticks are infective in the larval and nymphal stages; and of ovine piroplasmosis where larvæ and nymphs are infected and become infective in the adult stage.

Sarcocystis, the muscle parasite, occurs in many domestic animals including the pig, the horse, sheep and cattle, and it may assume considerable importance in certain localities. It also has been observed in birds and reptiles, and infections have been reported in man. Knowledge of the development and mode of infection with this organism is lacking in many important particulars.

These parasites most frequently are seen in the muscles of the skeletal system, but thorough search usually will discover them in the muscles of the œsophagus, larynx and diaphragm. They also may be found in the muscles of the eye and the tongue, and, occasionally, the heart. They occur as yellowish-white elongated bodies ranging in length from 15 to 50 or more millimeters. These are called "Miescher's tubes." They lie in the voluntary muscle fibres, which become more or less distended. In the late stages of the infection where much muscle tissue has been destroyed they lie in connective tissue.

These parasites are protected by a membrane. In the early stage of development this is very thin and homogeneous, but later it becomes thicker and radially striated. The body becomes chambered, and these chambers contain numerous falciform nucleated bodies, the spores or "Rainey's corpuscles." They vary in size and shape according to the species. The minute structure of these bodies has undergone varying interpretations at the hands of various writers.

It has long been known that sarcosporidiosis could be produced in mice by feeding them infected tissue. Fæces of an infected animal are said to be infective at times. Infection seems to take place through the intestinal epithelium where the parasite, according to Erdmann, appears to remain in the lymph spaces for about a month before passing to the muscles. Work of other investigators indicates that considerable time elapses before the parasites finally reach the muscles. The duration of life of the parasites is long, but in many cases they die in the tissue, becoming encapsulated. Sometimes calcification occurs as in the case of *Trichina* and Cysticercus. Those interested in details regarding the sarcosporidia are advised to have recourse to the current literature, for the subject is in such a confused state that it cannot be summarized here without adding to the confusion.

THE INFUSORIA

These are the most highly developed of the Protozoa. Their motile organs are in the form of flexible, vibratile protoplasmic processes known as cilia. These differ from flagella in being shorter. The stroke, also, is different, being sharp and quick in the propulsive movement and slower and less forceful on the recovery. In some forms these cilia may be fused to form cirri, membranelles and membranes. Most species have dimorphic

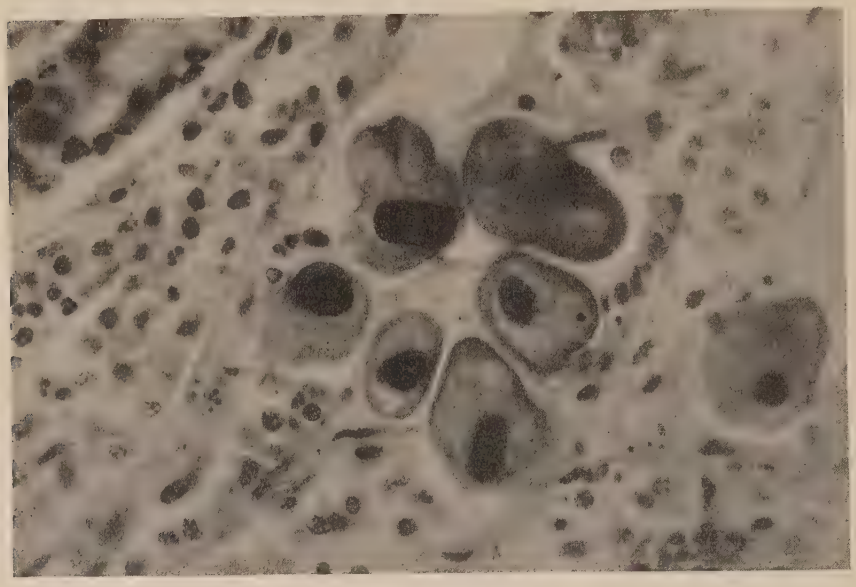


FIG. 66. BALANTIDIUM COLI

In colonic mucosa of Sus philippinensis. The parasites have worked their way towards the surface of the epithelium which has broken down as a result of the combined proteolytic and mechanical attack. Secondary bacterial invasion has supervened, the effects of which are evident. United States Army Medical Museum No. 38149. (Photomicrograph from preparation by Professor Haughwout.)

nuclei—that is to say, a macronucleus (meganucleus of some authors), that is concerned with the metabolic or vegetative processes in the organism and the micronucleus, a smaller body, which is active in the processes of reproduction and fertilization. Reproduction is by simple division or budding.

Of veterinary interest among the Infusoria are the large group of ciliates found parasitic in the stomachs of ruminants, including *Entodinium*,

Ophryoscolex, *Cycloposthium* and *Diplodinium*. In another sub-group occurs the genus *Balantidium*, of which two species have been described as occurring in the intestine of the pig.

Balantidium coli, the intestinal parasite of both man and the pig, has

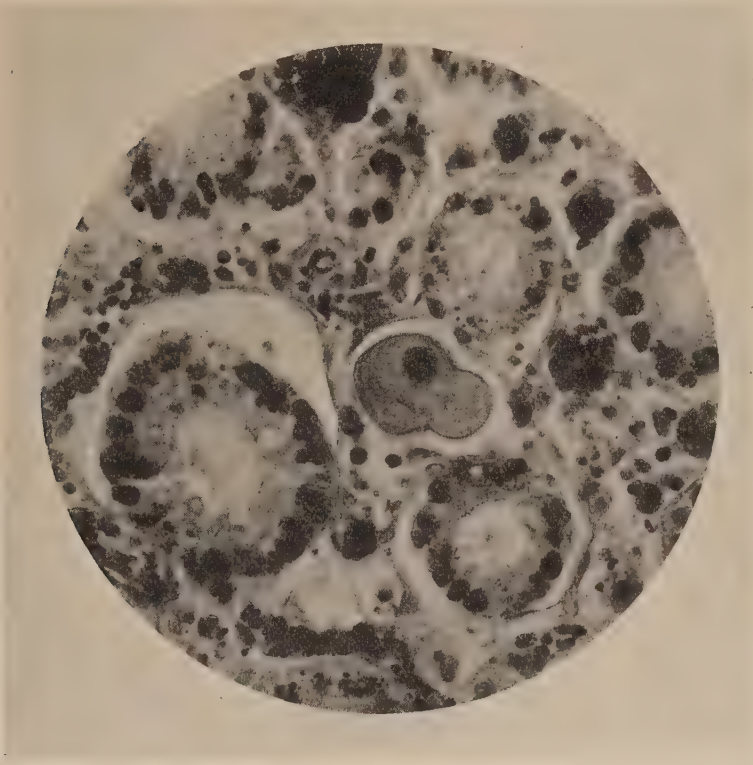


FIG. 67. BALANTIDIUM COLI

In stroma of colonic mucosa of Sus philippinensis. This material was from same source as that shown in Figure 66. The plasticity of the parasite is strikingly shown by the constriction and narrowing of the body. The nucleus is transected in the section. (Photomicrograph by Kelser from preparation by Professor Haughwout.)

been known to science since its description by Malmsten in 1857. It is only one of many species of *Balantidium* found in vertebrate and invertebrate hosts and recently McDonald[23] has added another species *Balantidium suis* found in pigs raised in the western United States. Whether this new

[23] McDonald, J. Daley, Univ. Calif. Pub. in Zoöl., xx, (1922), no. 10, pp. 243–300.

species has the same geographical distribution as *B. coli* remains to be determined. Up to the time of the publication of McDonald's paper all balantidia found in pigs appear to have been indiscriminately described as *B. coli*. With the exception of *Balantidium minutum*, a somewhat doubtful species established by Schaudinn, all balantidia of man have been regarded as *B. coli*. It is an obligatory parasite of the pig in which it habitually completes its life cycle including encystation, but it would appear to be incompletely adapted to man in that it seldom encysts in that host. Notwithstanding the statements of some authors, *Balantidium coli* is incapable of living a free aquatic life.

The question of the pathogenicity of *Balantidium* to pigs is clouded by the fact that most of the literature on the subject is very vague as to the species of pig involved. Most authorities adhere to the view that it is non-pathogenic to pigs, but the writer has evidence to prove that it is pathogenic to the black Philippine hog *Sus philippinensis* to the extent that it invades the tissues of the gut and can be found in large numbers in the mucosa and sub-mucosa, presenting a picture that is the replica of the infected gut in man. Figure 66 is a photograph of one of the writer's sections cut from the gut of a Philippine pig. Further work is needed to settle the question as to whether the parasite causes a colitis of the type seen in human beings infected with *Balantidium*. It is not impossible that it may cause dysentery in some varieties of pig and not in others.

McDonald describes *Balantidium coli* as encountered by him as follows:

> The individuals of the species *Balantidium coli* are ovoid in form, the more pointed end being anterior; length varies from 30 to 150μ; breadth varies from 25 to 120μ; in the majority of individuals the length is 1.3 times the breadth; the greatest transverse diameter intersects the longitudinal axis posterior to its midpoint; the adoral zone is approximately terminal, and the anterior tip of the body lies within it; the plane of demarcation between the apical cone of the ectoplasm and the endoplasm is approximately at right angles to the long axis of the body; the macronucleus is elongate but the length usually does not exceed three times the breadth; two contractile vacuoles are present, a smaller one located anteriorly and a larger one located posteriorly; a posterior cytopyge is usually distinctly visible.

He summarizes the specific characters of *Balantidium suis* thus:

> Body elongate; length approximately twice the breadth and varies from 35 to 120μ; breadth from 20 to 60μ; usually tapers more posteriorly, is blunter anteriorly, longest diameter transects longitudinal axis anterior to its midpoint; adoral region ventrally placed, cytostome one-fifth of way posteriorly along ventral surface; nucleus rod- or sausage-shaped, at least one-half the length of the entire organism, its width about one-fourth of its length; the species is parasitic in the pig.

McDonald goes on to say that he recovered these organisms from the pig *Sus scrofa*. He was unable to secure evidence as to the infectivity of this species of *Balantidium* to man.

There seems little reason to believe that these parasites are especially dangerous to pigs of the species common in the United States and Europe, but the writer does not consider that complete disposal of that point has been made. The incidence of infection of pigs with *Balantidium* appears to be high in all countries where the parasites have been studied. McDonald reports 68 per cent infected among the 200 pigs he examined.

The complete life cycle of this parasite has not been developed. Dividing forms frequently are encountered both in human and pig fæces. The writer never has been able to get the complete picture, most of the dividing forms encountered by him being in the meta- or early anaphase of division. The type of binary fission is that characteristic of the ciliata (transverse to the long axis of the organism). There is some evidence that conjugation occurs, but its details have not been worked out. According to McDonald, the active forms are found in greatest numbers in the region of the cæcum and may exceptionally be found in the lower end of the ileum. As the parasites are carried out of the area of fluid fæces and into the lower part of the gut where the fæces become formed, they encyst. The cysts are tough and difficult to stain.

Mention already has been made of the ciliates found in the stomachs of ruminants. Much information concerning them is contained in the paper on *Diplodinium ecaudatum*, by Sharp.[24]

TECHNICAL METHODS

Reference already has been made to certain principles that must be observed in the study of the Protozoa. These principles may now be briefly elaborated. Explicit examples cannot be given to cover all cases and the investigator is referred to the standard works on cytological technique, and the works of individual writers.

The main point to bear in mind is that the Protozoa are exceedingly delicate organisms. Degeneration, plasmolysis, desiccation and improper fixation leave their marks in distortion and the destruction of structural relations. It is very easy to produce cell artifacts by a little carelessness or the employment of faulty technique, and still easier to formulate erroneous interpretations on the evidence produced by such treatment.

Blood parasites such as *Piroplasma*, *Trypanosoma*, *Plasmodium* and

[24] Sharp, R. G., Univ. Calif. Pub. in Zoöl., xiii, (1914), 43–122.

Hæmogregarina, may be fixed and stained by the usual blood methods. The picture so produced will serve for identification and certain organelles such as flagella and undulating membranes are fairly well shown, but the method destroys the nucleus and obliterates delicate cytological detail. This method never should be used in the study of lumen- or tissue-dwelling parasites. The following cardinal principles should be observed:

1. The material must be *absolutely fresh.* That is to say, it must be fixed *immediately* after death if the best results are to be attained. Incredible as it may seem, a delay of a few minutes may lead to marked structural changes that will ruin precise work on the nucleus. Chilling of amœboid forms causes them to round out. In experimental work it always is possible to perform fixation promptly. In diagnostic work a brief delay is of less moment. The organisms will be recognizable but accurate cytological work may be impossible.

2. From the time material is placed in the fixing solution until it is sealed under the cover glass, it never must be allowed to dry. If, through accident, it dries, it should be thrown away for it is unfit for further manipulation.

3. The staining process should be controlled under the microscope, for a stain that demonstrates tissue elements in a satisfactory manner, may be either an overstain, or an understain of a protozoön. It may be necessary to make separate preparations to demonstrate certain organelles. It often happens that the complete structure of a protozoön cannot be shown satisfactorily in a single preparation.

It is assumed that the reader is familiar with the ordinary methods of histological technique, accordingly, superfluous details will be omitted.

Fixation

This may be accomplished by Zenker's fluid, Bouin's fluid or Schaudinn's fluid.

Zenker's Fluid.

Potassium bichromate	2.5 grams
Mercuric chlorids ($HgCl_2$)	8.0 grams
Distilled water	100.0 cc.

Add 5 per cent of glacial acetic acid at time of using.

This may be used for the fixation of either tissues or smears.

Fix tissues for 12 to 24 hours according to thickness. Wash in running water for 12 to 24 hours. Smears should be fixed for 10 to 15 minutes, and washed for 5 or 6 hours. Dehydrate, clear and embed in paraffine.

Sections and smears should be treated with iodized alcohol before staining to remove the excess of mercury and the iodine should be removed in clear 70 per cent alcohol. This is a good general fixative.

Bouin's Fluid.

Picric acid (saturated aqueous solution)	75.0 cc.
Formaldehyde (40 per cent)	25.0 cc.
Glacial acetic acid	5.0 cc.

This, also, may be used for either tissues or smears.

Fix tissues for 24 to 36 hours according to thickness. Transfer directly to 70 per cent alcohol. (Do not place material fixed in Bouin in water until it has been hardened in alcohol for at least 12 hours.) Change alcohol frequently until it no longer extracts a marked amount of picric acid. Smears should be fixed for about half an hour. (Overfixation does not occur with this reagent.) Care must be used to remove the excess of picric acid from tissue, otherwise it is impossible to section the material.

This is one of the best cytological fixatives. It is especially good for the demonstration of nuclear details and mitotic figures. After the removal of the picric acid the material is dehydrated and cleared in the usual manner.

Schaudinn's Fluid.

Mercuric chloride (saturated aqueous solution)	100.0 cc.
Ethyl alcohol (95 per cent)	50.0 cc.

Add 5 per cent glacial acetic acid at time of using.

This reagent should not be used for the fixation of tissue. For the study of free-swimming forms such as the lumen-dwelling species of the intestinal tract this is one of the best reagents we have. Smears should be fixed for 10 minutes, washed in 50 per cent alcohol, and treated with iodized alcohol for 10 or 15 minutes. The iodine should be removed in 70 per cent alcohol.

Smears may always be treated as if they were tissue sections. Slides should be free from grease and if the material does not adhere, one or two loopfuls of albumin may be mixed with the watery mass.

Staining

For the general run of work the hæmatoxylin stains will be found to give the best results. The writer customarily employs the methods of Delafield and M. Heidenhain for the demonstration of nuclear and acces-

sory structures and Congo Red, Bordeaux Red or Eosin in 0.5 per cent solution in 70 per cent alcohol for the demonstration of the cytoplasm and achromatinic structures. As has been said, nuclear staining should be controlled under the microscope. This is done by mounting the specimen in water under a coverglass, at intervals during the process of differentiation and stopping the extraction when the structures it is desired to demonstrate, stand out clearly.

Delafield's Hæmatoxylin.

Hæmatoxylin crystals	4.0 grams
Ethyl alcohol (95 per cent)	25.0 cc.
Saturated aqueous solution of ammonia alum	400.0 cc.

Dissolve the hæmatoxylin in the alcohol and add it to the alum solution. Expose to the air and light in an unstoppered bottle for 3 or 4 days. Filter and add—

Glycerine	100.0 cc.
Methyl alcohol (95 per cent)	100.0 cc.

Allow the solution to ripen in the light until it is dark, filter and transfer to a well-stoppered bottle. When it turns red it is unfit for use.

The writer makes up a staining solution according to the following formula:

Delafield's hæmatoxylin	20.0 cc.
Distilled water	80.0 cc.
Glacial acetic acid	5 or 6 drops

Sections or smears should be stained in this until nuclei are well shown. The counterstain may then be applied and the preparation mounted according to the usual method.

Heidenhain's Hæmatoxylin.

1. Mordanting and extracting solution

Ammonio-ferric alum	2.0 grams
Distilled water, q.s.	100.0 cc.

Discard the brown crystals, using only the purple crystals.

2. Stock staining solution

Hæmatoxylin crystals	1.0 gram
Ethyl alcohol (95 per cent)	10.0 cc.
Distilled water, q.s.	100.0 cc.

3. Staining solution

Stock staining solution..... 25.0 cc.
Distilled water..... 25.0 cc.
This makes sufficient staining solution (0.5 per cent) for an ordinary Coplin's jar.

Mordant smears or sections for 15 minutes in the iron-alum solution.
Wash 15 minutes in running water.
Stain until preparation is jet black.
Differentiate in mordant solution (under the microscope).
Wash in running water for 1 hour.
Dehydrate, clear and mount.

Flagella often are difficult to demonstrate, especially in permanent preparations. With care they can be shown in the hæmatoxylin preparations but usually at the expense of other cell structures.

Hogue[25] has recently devised a useful *intra vitam* method employing Janus Green B. She sets it forth as follows:

> A 0.5 per cent aqueous solution of janus green B was diluted with normal salt or distilled water according as to whether parasitic or free-living protozoa were to be studied. The dilutions used were one drop of 0.5 per cent aqueous solution to 10 or 20 drops of water. The stain was added either directly to the drop of culture and then covered with a cover glass, or it was placed at the side of the coverglass and drawn under by capillary attraction.

In the study of encysted protozoa in fresh fæces, the internal structures of the cysts are well shown by adding a drop or two of iodine solution (double strength Weigert) to the fæces before applying the coverglass. This often will demonstrate the nucleus and flagella of naked forms.

CONCLUSION

This concludes our brief excursion into the field of protozoölogy. It is the writer's belief that in a chapter such as this, the less said about the structure of individual species of protozoa, the better. The literature on the protozoa is rich, and some of the best works are available in the large cities or easily obtained from the publishers. Among the best of the text-books the writer would mention those of Minchin,[26] Doflein,[27] Brumpt,[28]

[25] Hogue, M. J., Stain Technology, i, (1926), 35–36.

[26] Minchin, E. A., An Introduction to the Study of the Protozoa, with Special Reference to the Parasitic Forms, (1912), London, Edward Arnold.

[27] Doflein, F., Lehrbuch der Protozoenkunde, (1916) 4e, Jena, Gustave Fischer.

[28] Brumpt, E., Précis de Parasitologie, (1913) 2e, Paris, Masson et Cie.

and Wenyon.[29] These should prove helpful in routine work to the man in search of information concerning the common species. The files of The Tropical Diseases Bulletin and The Tropical Veterinary Bulletin contain admirable reviews of current literature.

In conclusion, the writes makes bold to offer a few suggestions to the men who, like himself, have had tasks laid upon them for which they lacked the special training requisite to an effective attack.

1. Beware of the pitfalls of cellular pathology.

2. Interpret so-called "sexual phenomena" in the protozoa with caution. Mistakes of the most incredible absurdity have been made by even accomplished workers. Study the nucleus carefully in forms that are believed to be dividing.

3. Interpret the presence of parasitic protozoa in a given locality in the body in terms of the type of nutrition shown by the species in question and its mode of life. Be watchful for contaminating non-parasitic species.

4. View askance hyaline or non-nucleated bodies, or bodies that do not respond to the usual staining technique, aside from frankly encysted forms. All protozoa are nucleated and all, under proper technique exhibit a definite protoplasmic structure of one kind or another.

5. Distinguish carefully between the cellular and tissue reactions of an inflammatory nature, that frequently arise from bacterial invasion of the tissues, and the non-inflammatory reactions produced by the protozoa; and be on guard against too hasty conclusions concerning "toxæmia" in protozoal infections. Remember that protozoal infections tend toward chronicity.

6. The mere presence of a protozoön in the lumen of an organ or, in some instances, even within cells or tissues does not stamp it as a lethal parasite or even as the cause of some acute process that may be present.

7. Work hand in hand with the pathologist in all questions involving the pathogenicity of protozoa.

8. In short, attempt no ill-considered flights into the realm of protozoölogy.

9. Above all, leave the naming and systematic placing of all "new" protozoa to the specialist. He may have seen or heard of the organism in question before, the literature accordingly will escape one more incongruous synonym.

[29] Wenyon, C. M., Protozoology: a Manual for Medical Men, Veterinarians and Zoologists. 2 vols. (1926) London, Baillière, Tindall and Cox.

CHAPTER XXXIV

Sarcodina

As indicated in the preceding chapter the Sarcodina are protozoa which move about by throwing out protoplasmic processes known as pseudopodia.

From a medical standpoint there is one genus of this Class which is of particular importance—the genus Entamoeba. In so far as veterinary medicine is concerned, this same genus is of interest. It is, however, only of minor importance because disease among the lower animals, due to amoebae, is exceedingly rare.

Amoebic dysentery in dogs and cats has been occasionally described by various authors. In some such instances the parasite is described as resembling Entamoeba histolytica, the cause of amoebic dysentery in man, while in other cases similarity to Entamoeba coli is noted.

There are several reports of outbreaks of amoebic dysentery among monkeys. One such outbreak was carefully studied by Eichhorn and Gallagher[1] in 1916. While the organism they studied was found to be somewhat similar to Entamoeba histolytica they concluded it was a different species.

In 1895 Theobald Smith[2] reported finding in cases of entero-hepatitis (blackhead) of turkeys, an organism which he designated "Amoeba meleagridis." As a result of this and later work Smith definitely concluded that such "amoeba" was the cause of the disease. Subsequent studies are confirmatory of Smith's findings as to the organism observed being the cause of the disease. However, the conclusion that the organism is an amœba has been questioned by several investigators, particularly Hadley.[3] Evidence has been brought forth which quite definitely proves that the blackhead organism is not an amoeba but a Trichomonas—Trichomonas meleagridis.

The structure and characteristics of the amoebae have already been dealt with in the preceding chapter.

[1] Eichhorn and Gallagher, Jour. of Infectious Diseases, xix, no. 3, 1916.

[2] Smith, Theobald, U. S. Dept. of Agric., B. A. I. Bull. No. 8, 1895.

[3] Hadley, Rhode Island Agric. Exp. Sta. Bull. No. 166, June, 1916, and No. 168, November, 1916.

CHAPTER XXXV

Mastigophora

The mature protozoa of this class are characterized by the presence of one or more whip-like flagella. There are three genera containing pathogenic species of interest in veterinary medicine. The first to be considered is *Trypanosoma* and is the most important genus. The genus *Leishmania* contains one species of importance to veterinarians. The third genus is *Trichomonas* and is briefly discussed because a species of same is apparently the etiological factor of enterohepatitis of turkeys.

GENUS TRYPANOSOMA

The genus Trypanosoma consists of a rather large number of species some of which are pathogenic for man and lower animals, producing diseases of serious character.

Valentine (1841) is commonly credited as being the first to observe trypanosomes. It is stated that he found such organisms in trout. It is very doubtful that the organisms Valentine observed were actually trypanosomes. The term "trypanosoma" was first employed by Gruby in 1844 to designate a flagellated organism he had noted two years before in the blood of frogs. Such organism was undoubtedly a true trypanosome.

In 1878 Lewis, working in India, discovered the now well-known, nonpathogenic, rat trypanosome which Kent later named "Trypanosoma lewisi." Two years later (1880) Griffith Evans, also working in India, discovered the surra trypanosome which we now know as "Trypanosoma evansi." This discovery was of immense importance as it constituted the first recognition of the ability of trypanosomes to cause disease. Previous to that time protozoa of this genus were looked upon as harmless parasites.

A number of the trypanosomes have been cultivated on artificial culture medium, particularly that of Nicolle, Novy and MacNeal (N. N. N. medium). However, growth is usually very difficult to obtain, particularly with some species.

The species of trypanosomes of importance in veterinary medicine follow:

Trypanosoma evansi

As mentioned above Trypanosoma evansi, the causative agent of surra, was discovered by Griffith Evans and was the first pathogenic species observed.

Surra has been reported from India, Indo-China, Burma, Philippine Islands, Persia, Java, Sumatra, Mauritius and Africa.

The disease is a fatal trypanosomiasis, particularly of the equine species. Cattle and carabaos (water buffalo) are occasionally affected but the disease in such animals is seldom of serious consequence, the symptoms usually being mild. However, in countries where surra exists cattle,

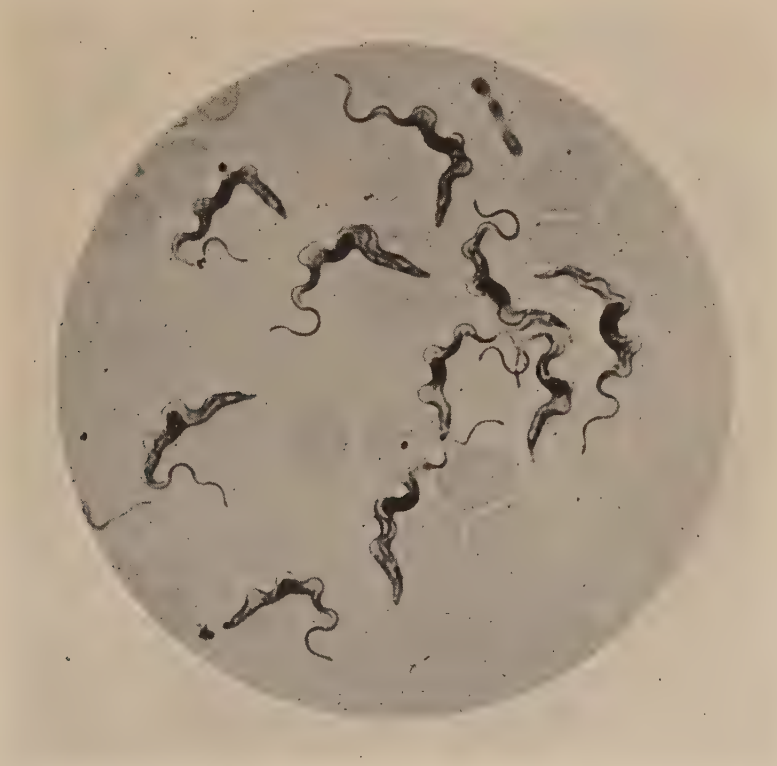


FIG. 68. TRYPANOSOMA EVANSI

carabaos and various wild animals are important factors as carriers. The author is, at the present writing, studying the carrier problem in surra in the Philippine Islands. Several hundred carabaos and a similar number of cattle in surra districts have been examined. The procedure has been to subject blood specimens from such animals to the complement-fixation test for trypanosomiasis and then prove the type of trypanosome carried

by reactors by animal inoculation tests. Over 50 per cent of the carabaos and more than $33\frac{1}{3}$ per cent of the cattle examined to date have been found to harbor Trypanosoma evansi. Camels are readily susceptible to surra. Dogs, particularly hunting dogs, are occasionally affected.

Morphology. Trypanosoma evansi is a fairly large trypanosome, the average measuring from 25 to 30 microns in length and 1 to 2 microns in breadth. It possesses a well-developed, undulating membrane and a rather long, free flagellum. The nucleus is oval in shape and situated approximately in the center of the cell. The parabasal body (kinetonucleus) is usually round and rather small. The anterior extremity (flagellar end) is pointed whereas the posterior extremity is fairly blunt. A few deep-staining granules are observed in the endoplasm of some of the parasites. The organism is actively motile, the movement being of a very flexible, serpentine character.

Transmission. The surra trypanosome can be transmitted by flies, particularly Tabanidae. Flies of the genus Stomoxys have also been incriminated. The author has been unable, experimentally, to transmit surra through the agency of Stomoxys calcitrans. Mitzmain and others likewise failed to find Stomoxys calcitrans a factor in the transmission of surra in the Philippines.

In laboratory experiments the writer has mechanically transmitted Trypanosoma evansi from infected to normal white rats through the agency of the mosquito Aedes egypti. The mosquito, however, is probably not the usual factor in the spread of the disease. In a series of experiments about to be published the writer has demonstrated that the common transmitting agent of surra among horses in the Philippines is the fly Tabanus striatus. This is in accord with Mitzmain's findings published some years ago.

In dogs there is evidence to indicate that ticks and possibly fleas are factors in transmission of the disease.

Dogs are reported susceptible to infection through the alimentary tract. When such type of infection occurs it is undoubtedly due to the introduction of the trypanosomes into the tissues through minor injuries.

Pathogenicity. Rats, mice, guinea pigs and rabbits are susceptible to infection with Trypanosoma evansi. In rats and mice the parasites appear in the circulation in about 60 to 72 hours after inoculation and constantly increase in numbers without remission so that within 5 or 6 days the blood is swarming with the flagellates. Such animals usually succumb in 7 or 8 days.

When guinea pigs and rabbits are inoculated the trypanosomes are

usually not present in the circulation in detectable numbers until after about 10 days. Then, in these animals, the presence of the parasite in the blood is subject to considerable fluctuation. One day a large number of trypanosomes are present while on the following day examination may reveal only a very limited number or even an entire absence of the flagellates. Infected guinea pigs will usually survive one or two months, sometimes longer. Rabbits will frequently survive 3 or 4 months.

Serological Reactions. Animals affected with surra readily respond to the complement-fixation test for trypanosomiasis. In acute cases of surra in equines and camels microscopic examination of fresh blood preparations is frequently sufficient to establish a diagnosis. However, as the presence of the parasite in the circulation is subject to fluctuation during the course of the disease the complement-fixation test can sometimes be used to advantage.

The greatest value of the complement-fixation test is in connection with the identification of carriers among cattle, carabaos, etc. Microscopic examination for the detection of carriers will, in the vast percentage of cases, fail to identify same. The trypanosomes are rarely present in the circulation of carriers in considerable numbers except at irregular periods, principally at times when the normal resistance of such animals is lowered from some other cause. Such carriers, however, will react to the complement-fixation test and if a quantity of their blood is injected into several rats or guinea pigs the trypanosome can be recovered.

The complement-fixation reaction is a group phenomenon. In other words, an animal infected with any one of the trypanosomes will respond to the test irrespective of the type of trypanosome entering into the preparation of the antigen. For this reason, positive reactors among apparently healthy carriers should be followed by animal inoculation tests to rule out the possibility of the reaction being due to a non-pathogenic variety of trypanosome. Rats are best suited for such inoculation tests. Where several types of pathogenic trypanosomes are present in a locality, the variety harbored by carriers is, obviously, not indicated by the serological test.

For details of the complement-fixation test for trypanosomiasis see section on Serology.

Immunization. There is no known method for the immunization of animals against surra.

Trypanosoma brucei

Trypanosoma brucei was discovered in 1894 by Bruce in Zululand and demonstrated to be the etiological factor of nagana. This disease is

characterized by remittent fever, edema, marked weakness and emaciation The disease occurs in horses, cattle, sheep, goats and dogs. In the equine species it runs an acute and fatal course. In cattle it frequently assumes a chronic form and a small percentage of the animals recover. The disease is known to exist only in Africa.

Morphology. Trypanosoma brucei is practically identical in its morphology, etc., with Trypanosoma gambiense, the causative factor of African sleeping sickness in man. As a matter of fact the evidence is strong that these two trypanosomes are one and the same species. This trypanosome, in a general way, resembles the surra parasite. It is not quite as slender as Trypanosoma evansi. The posterior extremity is possibly a little more blunt and the flagellum somewhat shorter. Trypanosoma brucei usually appears to contain more metaplastic granules and is not as actively motile as the surra parasite. It should be emphasized that these possible differences are by no means well marked or outstanding.

Transmission. Trypanosoma brucei is transmitted by flies particularly the tsetse fly (Glossina palpalis). The parasite is harbored by various wild animals (water buffalo, zebras, antelopes, etc.) in the infected districts.

Cultivation. Trypanosoma brucei has been propagated on artificial media but its culture only occasionally succeeds.

Pathogenicity. Rats, mice, guinea pigs, rabbits and dogs can be employed for inoculation purposes. The behavior of the parasites in these animals is similar to that of the surra trypanosome.

Serological Reactions. Complement-fixation reactions can be readily obtained with serum specimens from animals suffering from nagana and from those acting as reservoirs.

Trypanosoma equiperdum

Trypanosoma equiperdum is the etiological agent of dourine or as the French term it "mal du coit." This is a chronic malady of stallions and brood mares, characterized by edema, especially about the external genitals, and subsequent involvement of the nervous system. The causative trypanosome was discovered in 1896 by Rouget.

Dourine occurs in various parts of Europe and Asia and to a very limited extent in the United States. It first appeared in the United States in 1901 but through the efforts of the United States Department of Agriculture it was apparently eradicated by 1906. In 1912 the affection was again diagnosed in Montana. The investigation which followed revealed the infection in ten states. Energetic measures for the control and eradication of the disease were inaugurated by the Federal government in co-

operation with the authorities of the states concerned. As a result of these efforts the disease in the United States has been practically eradicated. In this connection, it should be mentioned that the results obtained were made possible largely through the utilization of the complement-fixation test for the diagnosis of the disease.

Morphology. The dourine trypanosome resembles Trypanosoma brucei very closely. One feature worthy of mention is that when observed in preparations obtained directly from affected horses, Trypanosoma equiperdum has never been observed to possess metaplastic granules.

This trypanosome is seldom found in the circulating blood. In making

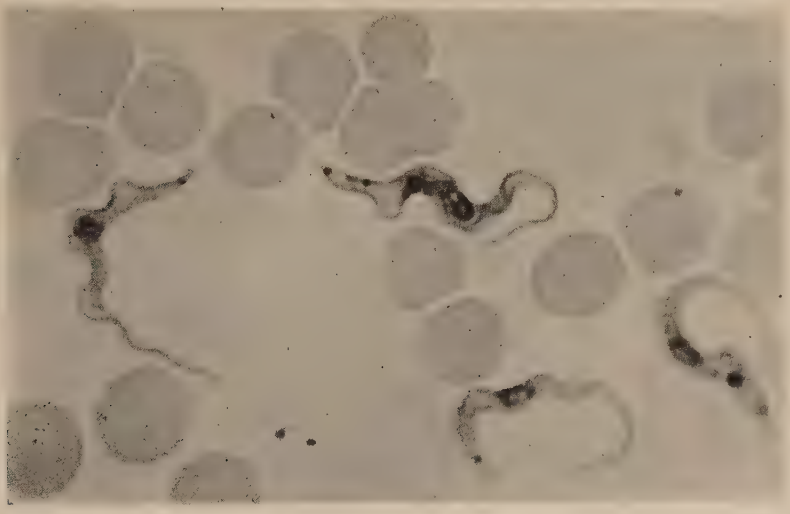


FIG. 69. TRYPANOSOMA EQUIPERDUM
(Photomicrograph by Maj. G. R. Callender, Army Medical Museum)

microscopic examinations it is best to employ serous fluid taken from dourine "plaques" or from edematous swellings about the genitals. The examination of uretheal or vaginal secretions frequently reveals the trypanosome.

Transmission. Unlike the other trypanosomiasis dourine is commonly transmitted from animal to animal through coitus.

Cultivation. The cultivation of Trypanosoma equiperdum has been reported but it is an extremely difficult task, most attempts resulting in failure.

Pathogenicity. Rats, mice, guinea pigs and rabbits can be readily in-

fected by injecting them with material containing Trypanosoma equiperdum.

Trypanosoma equinum

Trypanosoma equinum is the cause of a South American disease of horses, known as "mal de caderas." The trypanosome was identified as the cause of the disease in horses in Argentina in 1901 by Elmassian.

Mal de caderas is a very common malady of horses in some sections of South America. The disease occurs as a subacute or chronic affection characterized by slight intermittent fever, marked weakness, particularly of the hind quarters, emaciation and usually a marked conjunctivitis. As the disease advances paralysis of the hindquarters becomes more marked and characterizes the affection.

Morphology. Trypanosoma equinum differs but slightly from those previously referred to. Its parabasal body, however, is very minute and difficult to demonstrate by staining methods. The organism is very active, much more so than the nagana trypanosome.

During the acute stages of the disease the trypanosome can usually be demonstrated microscopically in rather large numbers in the circulating blood. There are frequent remissions, however, so that the parasite cannot always be found. Animal inoculation tests will frequently demonstrate the trypanosome when microscopic examination fails. Rats and mice can be utilized for such purposes.

Transmission. The exact mode of transmission of Trypanosoma equinum is not definitely known. Flies have been suspected but numerous investigators have failed to definitely incriminate them as disseminating agents. A number of authorities believe that the disease is transmitted to horses by parasites, possibly fleas, which have previously infested the capybara, a rodent which frequently becomes affected with a malady thought to be due to Trypanosoma equinum. There is experimental evidence which indicates that infection can be brought about through the alimentary canal. Dogs frequently become infected through feeding on carcasses of animals dead of mal de caderas.

Trypanosoma hippicum

In 1910 Darling, in Panama, demonstrated Trypanosoma hippicum in the blood of horses and mules suffering from a usually fatal disease known as "murrina." The malady is characterized by progressive anemia, edema and emaciation. The disease is not communicable to cattle.

Morphology. Trypanosoma hippicum is 18 to 28 microns in length and

1.5 to 3 microns wide. The parabasal body is near the aflagellar end and stands out very clearly, a point which is emphasized as characteristic of the organism. The endoplasm usually contains numerous, rather coarse, metaplastic granules.

Transmission. Flies are probably responsible for dissemination of the disease although some investigators believe that it can also be transmitted through coitus.

Pathogenicity. Rats, mice, guinea pigs and rabbits are susceptible.

Other Trypanosomes

A number of very closely related trypanosomes have, from time to time, been reported as the etiological agents in various conditions among lower animals in Africa. It is highly probable that some of these species are identical and, further, that they may not all be pathogenic.

Trypanosoma dimorphon. This trypanosome is the causative factor of a chronic disease of horses known as "gambia fever." The malady is characterized by intermittent fever, weakness, edema, especially about the genitals, and emaciation.

Trypanosoma dimorphon is found in the early stages of the disease as a short, tadpole-like form. As the disease gets well under way the organism changes so that it appears as a short, thick, stumpy form with a short flagellum. Shortly before the disease terminates fatally the trypanosome is observed as a long, slender form with long flagellum. Some authorities regard the different types as neuter, female and male forms.

Flies, particularly those of the genus Glossina, are believed to be transmitting agents.

Rats, mice, guinea pigs and rabbits can be infected with this organism.

Trypanosoma cazalboui. This trypanosome resembles the surra organism. It is reported as the cause of a septicemic affection of horses, cattle and sheep known as "souma."

Trypanosoma cazalboui differs from most of the other pathogenic types in that it is practically avirulent for dogs and laboratory animals.

The disease is thought to be transmitted by flies.

Trypanosoma pecaudi. A disease known in Africa as "Baleri" among horses, cattle and sheep is due to this trypanosome. The malady usually terminates fatally in horses whereas in cattle and sheep it runs a mild course.

The long forms of this trypanosome possess free flagella whereas a second short and broad type does not have a flagellum extending beyond its cell structure.

Transmission is thought to be by flies of the genus Glossina.

Dogs and laboratory animals are susceptible.

Trypanosoma congolense. Described by Broden in 1904 as the cause of a nagana-like disease in the French and Belgian Congos and in Northeast Rhodesia.

The organism possesses no free flagellum, resembling the aflagellar form of Trypanosoma dimorphon.

Trypanosoma theileri. Theiler found this large trypanosome in the blood of cattle suffering from "gall-sickness" or "galziekte" in South Africa and believed it to be the cause of the disease. Subsequently he determined that the organism was a non-pathogenic parasite.

This trypanosome can only be propagated in the bovine species.

Trypanosoma lewisi. While this is a non-pathogenic organism it is briefly mentioned for several reasons. First, it is a very common parasite in wild rats practically in all sections of the world. It is, therefore, easily obtained and, being non-pathogenic, is often used in class work. Then, most of the life history of this trypanosome has been satisfactorily worked out. For this latter knowledge we are indebted to Minchin and Thomson.[1] The trypanosome passes part of its life-cycle in the rat flea (Ceratophyllus fasciatus) and occasionally in the rat louse (Haematopinus spinulosis). In the flea a phase is passed in the stomach and another in the rectum. The flea disseminates the infection through regurgitation of material containing small forms of trypanosomes or through passing them with the feces.

Trypanosoma lewsi measures 28 microns long and 1.5 to 2 microns broad. It possesses a pointed posterior extremity and has an oval or rod-shaped parabasal body. Its nucleus is situated towards the anterior end. Trypanosoma lewisi possesses a very rapid movement which is not as flexible in character as that noted with a number of the other trypanosomes. The undulating membrane of Trypanosoma lewisi is comparatively narrow.

So far as is known, Trypanosoma lewisi cannot be propagated in any animal other than the rat. For several weeks following infection of this

[1] Minchin and Thomson, Quart. Jour. Micro. Science, lx, 1915.

rodent there is a progressive increase in the number of trypanosomes. They then gradually disappear leaving the animal immune.

GENUS LEISHMANIA

The genus Leishmania is composed of several organisms which occur in the body of the host as small, round or oval bodies containing a nucleus (macro- or tropho-nucleus) and a parabasal body (micronucleus or blepharoplast). In culture they are found to possess a flagellum, similating a trypanosome without an undulating membrane.

The first organism of this genus was described by Leishman in 1903. He observed in spleen specimens from cases of what was known as "dum-dum" fever (kala-azar) in man, in India, the organism now commonly termed "Leishmania donovani." Leishman at first considered the organism as a type of trypanosome. Shortly following Leishman's report Donovan, independently, described the observation of similar bodies in spleen specimens from cases of kala-azar, which disease, he, at that time, termed "malarial cachexia." The genus was named "Leishmania" by Ross and the species responsible for kala-azar in man designated "Leishmania donovani."

In 1909 Nicolle described, in cases of infantile splenomegaly or infantile splenic anemia, what he believed to be another species of Leishmania, terming the same "Leishmania infantum." At present there is sufficient evidence to indicate that this organism is not a separate species but is identical with Leishmania donovani. Further, it is now considered that the so-called "Leishmania canis," formerly recognized as being very similar to Leishmania infantum, is undoubtedly the same as the organism found in infantile splenomegaly. Thus, as the matter now stands, we recognize one organism, Leishmania donovani, as the cause of both adult and infantile kala-azar and canine leishmaniasis.

In man Delhi boil (oriental sore) is caused by Leishmania tropica. While this organism is practically indistinguishable from Leishmania donovani, it is probably a different species.

Leishmaniasis in dogs has been observed in Africa, Italy, Spain, Sicily, Malta and Greece. It is probable that in many instances the dog acts as a reservoir of the infection, man subsequently becoming infected through the agency of insects, probably fleas.

Leishmania donovani is found in the endothelial cells of the spleen, liver, bone marrow and lymph glands and to a lesser extent in the leucocytes of the peripheral circulation. Splenic puncture is frequently necessary to obtain satisfactory material for diagnostic purposes.

Leishmania donovani

In tissue preparations Leishmania donovani appears as a round or oval, non-motile cell, 2 to 4 microns in diameter when appearing round, and about the same length and 1.5 to 2.5 microns broad when oval in form. When stained with Giemsa's or Wright's stain the cytoplasm shows up very faintly and is found to contain a nucleus and parabasal body. The nucleus is oval and stains less intensely than the smaller, rod-shaped parabasal body. The nucleus and parabasal body are situated opposite each other near the periphery of the cell.

Leishmania donovani can be cultivated in sterile spleen extract containing 10 per cent sodium citrate solution. It can also be cultivated on "N.N.N." medium. Cultures should be incubated at a temperature of 20° to 22°C. When grown artificially the organism becomes elongated and develops a flagellum, appearing like a trypanosome minus its undulating membrane.

GENUS TRICHOMONAS

The trichomonads are pear-shaped flagellates commonly found in the intestinal tracts of man and the lower animals. The cell is rounded at its anterior end, tapering posteriorly to a point. Three to five long, anterior flagella are present, frequently observed matted together. An undulating or vibratory membrane starts at the point where the flagella emerge and extends backward. This is bordered by a fourth or posterior flagellum which continues as a free process beyond the point where the vibratory membrane stops. Running through the central portion of the cell, there is seen a somewhat curved, supporting structure known as an "axostyle." The nucleus of the organism is located in the anterior end. A number of vacuoles are found in the cell.

The organisms of this genus have been generally regarded as nonpathogenic. The evidence, however, is fairly definite that the species of Trichomonas observed in enterohepatitis of turkeys is of etiological significance.

Trichomonas meleagridis

As previously indicated, Trichomonas meleagridis is the organism first observed by Theobald Smith in cases of enterohepatitis (blackhead) of turkeys and mistaken by him for an amoeba ("Amoeba meleagridis"). The exact nature of the parasite was established largely as a result of Hadley's investigations. He demonstrated that the organism described by Smith, while in some respects resembling an amoeba, was actually the

late trophozoite (rounded) stage of a Trichomonas commonly found in the intestinal tract of turkeys and other fowl.

Hadley emphasizes the point that the Trichomonas is only capable of producing disease after the way has been paved by some unknown factor. Graybill[2] has indicated that Heterakis gallinae, a small thread worm commonly found in the ceca of turkeys and chickens is capable of preparing the way for invasion of the tissues by the blackhead organism. It was found that by feeding a considerable number of fully developed eggs of Heterakis gallinae to turkeys having the blackhead flagellates in their intestines the disease could be produced.

Trichomonas meleagridis is quite variable in shape, depending upon the stage of development. Some variation in form is also observed among individuals in the same stage of development. The characteristic and most common form is the pear-shaped organism. Older, free-swimming forms are apt to be more irregular in outline, appearing elliptical, crescent-shaped or ovoid. In the later stages the organisms appear round. In the free-swimming stage Trichomonas meleagridis measures 8 to 12 microns in length and possesses the basic characteristics of the genus which have already been mentioned.

The common mode of reproduction in the cecal contents is by simple fission. The usual mode of reproduction in the tissues is by encystation and spore formation.

[2] Graybill, Jour. Exp. Med., xxxi, 1920.

CHAPTER XXXVI

Sporozoa

The Sporozoa are all parasitic organisms which, with few exceptions, reproduce by spore formation. Organisms of this class are further divided into two groups—Telosporidia and Neosporidia. The Telosporidia are organisms in which the life of the individual ends with sexual spore formation. In the case of Neosporidia spore formation does not end the life of the individual, but may occur during the active life of the parasite without interfering with the vegetative processes.

Among the Telosporidia the Haemosporidia (Babesia, Theileria and Anaplasma) and the Coccidia (Eimeria and Isospora) are of interest in veterinary medicine.

Of the Neosporidia there is but one genus of interest to the veterinarian —Sarcocystis.

GENUS BABESIA

This genus contains several species of importance in veterinary medicine. The most important, however, is Babesia bigemina, the causative factor of Texas fever of cattle.

Babes,[1] in 1888, reported the finding of inclusions in red blood cells of cattle suffering from Texas fever. He failed, however, to recognize the true nature of the parasite but believed it to be a special type of bacterium. Five years later (1893) Smith,[2] at first independently, and later in association with Kilborne, reported his epoch-making findings on the cause and transmission of Texas fever, a contribution which will always stand out as a classic.

The genus is designated "Babesia" after Babes but it is also known as "Piroplasma." The diseases caused by the various members of the genus are commonly known as "piroplasmoses."

Babesia bigemina (*Babesia bigeminum, Piroplasma bigeminum*)

Texas fever is the common name applied to bovine piroplasmosis, caused by Babesia bigemina. The disease is also referred to as "tick fever,"

[1] Babes, Comptes Rendus des Séances de l'Academie des Sciences, cvii, 1888.
[2] Smith and Kilborne, U. S. Dept. of Agric. B. A. I. Bull. No. 1.

"red water," "black water," "hemoglobinuria," "bovine malaria," etc. In the United States the disease has been limited to the southern section of the country. Through the energetic measures for the eradication of the cattle tick, carried out by the Federal Bureau of Animal Industry in conjunction with the various States concerned, Texas fever in this country has been eradicated from a large portion of the originally infected section and the next few years will undoubtedly witness its entire eradication.

There is evidence which tends to indicate that bovine piroplasmosis in various sections of the world may not be due to the identical species of parasite. In European "red-water" of cattle, caused by Babesia bovis, the piroplasma appears smaller than Babesia bigemina and pear-shaped forms are less frequent. Further, recovery from one type of infection does not confer immunity against the other type.

Morphology. Piroplasma bigemina is found in the red blood corpuscles usually in pairs. They are generally pear-shaped with the pointed ends touching or approximating each other. They average 2 to 4 microns in length and 1.5 to 2 microns thick at the broadest point. Occasionally more than two parasites are observed in a single cell.

In addition to the pairs of pear-shaped parasites, a smaller round or ring form is observed, frequently occurring singly in the red blood cells. This latter form is not, however, invariably found singly as cells containing two parasites are not rare and occasionally 3 or even 4 are observed in a single blood cell. Besides the pear-shaped and round forms, various types of irregular forms are frequently observed.

In the broad end of the pear-shaped forms, near the periphery, a small, refractile body 0.1 to 0.2 micron in size is observed. In stained preparations this body takes the color rather intensely. Then more centrally located in the broad portion of the parasite a larger, round or oval body 0.5 to 1 micron in diameter is frequently demonstrable.

In fresh preparations some of the parasites show a type of amoeboid motion, the movement being due to a change in the contour of the parasite and not to the formation of definite pseudopodia.

Smith and Kilborne have indicated that up to within a short time before death only about 1 per cent of the red blood cells are found to contain the organism. Shortly before death 5 to 10 per cent are found infected.

Babesia bigemina can be demonstrated with several of the ordinary aniline dyes (Loeffler's methylene-blue, fuchsin, etc.) but for the best preparations Wright's or Giemsa's blood stain should be used.

Resistance. Blood from animals in the acute stages of the disease, when

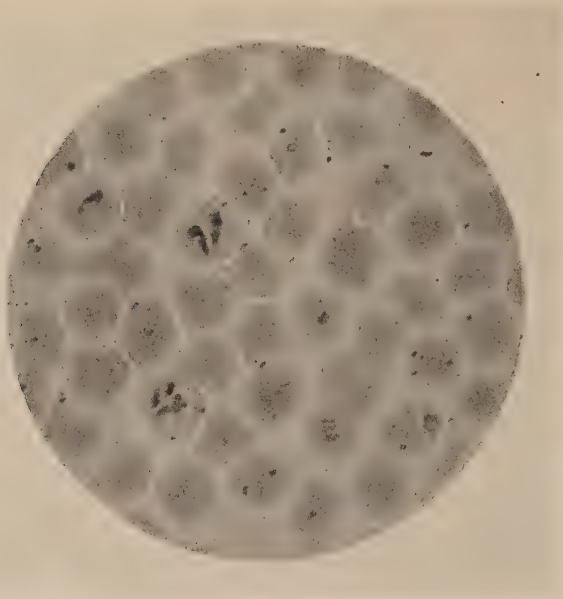


FIG. 70. BABESIA BIGEMINA

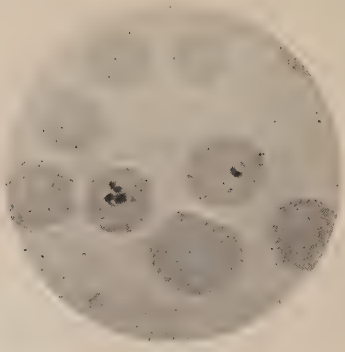


FIG. 71. BABESIA BIGEMINA (RING FORMS)

defibrinated and kept at a low temperature (6° to 10°C.) will remain virulent for one to two months. Moderate temperatures, drying, etc., soon result in the destruction of the organism.

Transmission. Babesia bigemina, in the United States, is commonly conveyed by the tick "Margaropus annulatus." Closely related species of ticks have been incriminated as intermediate hosts in various parts of the world. In northern and central Europe the common carrier is Ixodes ricinus.

The female tick engorges herself with blood from an infected animal and drops to the ground where she lays her eggs (2000 to 5000). In from 18 days to several months, depending upon temperature and other weather conditions, the eggs hatch and the resulting seed ticks soon climb from blades of grass, weeds, etc., on to passing cattle. They start to suck blood and in the process infect their host. In the case of Margaropus annulatus the different molts take place on the host. With some species of ticks (Ixodes ricinus) the molting takes place on the ground. The seed tick develops into a nymph and this in turn undergoes another molt and appears as the sexually mature tick. When originating from eggs of an infected female the ticks are infectious in all stages of their development.

Immunization. Cattle which have passed through an acute attack of piroplasmosis are subsequently immune. However, they continue to harbor the parasite in variable numbers in their blood for long periods of time.

The blood of recovered animals, containing but few parasites, can be utilized to immunize susceptible cattle. Inoculation of susceptible animals with such blood usually results in the development of only a mild form of the disease from which the larger percentage of cattle recover and are subsequently immune. In some instances, however, the losses from this method of immunization are rather high.

In place of blood from recovered adult cattle, blood from calves raised in Texas fever districts can be employed for immunization purposes and is probably less likely to result in heavy losses.

Lignières conducted experiments in South America in which he utilized heated blood from Texas fever cases for immunizing purposes. He reported quite satisfactory results.

Babesia equi (*Piroplasma caballi*, *Piroplasma equi*, *Nuttallia equi*)

Equine piroplasmosis has been reported from Africa, Russia, Italy, India. Venezuela and Panama. Horses, mules, donkeys and zebras are susceptible.

Gugliemi,[3] in 1899, discovered the parasite in the blood of affected horses

[3] Gugliemi, Clin. Vet., 1899, p. 220.

in Italy. Between 1901 and 1912 Theiler[4] working in South Africa, published a number of valuable accounts of equine piroplasmosis which added considerably to our knowledge of the subject.

Morphology. Babesia equi is found in the red blood cells of affected animals in the form of pear-shaped bodies, ring forms, coccoid bodies and rosettes of four ovoid structures. Some authorities group these various forms under two separate genera. The typical pear-shaped forms have been considered as belonging to the genus Babesia (Piroplasma) and are referred to as "Babesia equi" while the smaller, round forms and rosettes are grouped under the genus "Nuttallia" and are designated "Nuttallia equi." While it is barely possible that these are members of two different genera it is more probable that they are merely different forms of the same genus and species. While often but one type of the parasite can be found in a

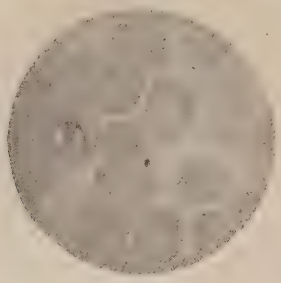


FIG. 72. BABESIA EQUI

particular case frequently both types are found. Further, the disease produced is identical, irrespective of the type of organism observed in the blood cells.

The pear-shaped forms are found in the blood cells usually in pairs, sometimes in fours and occasionally singly. The organism resembles Babesia bigemina but is a trifle smaller than same.

Transmission. Babesia equi is transmitted by the ticks Rhipicephalus

[4] Theiler, Schweizer Archiv. für Tierheilkunde (Zürich), 1901, xliii, p. 233.

Theiler, Deutsche Zeitschrift für Tiermedizin und Vergleichende Pathologic (Leipzig), 1904, viii, p. 382.

Theiler, Jour. Comp. Path., 1905, xviii, p. 229.

Theiler, Jour. Comp. Path., 1906, xix, p. 283.

Theiler, Rpts. of Govt. Bact., South Africa, 1905, 1906, 1907, 1908.

Theiler, Zeitschrift für Infektionskrankheiten, Parasit. Krankheiten und Hygiene der Haustiere (Berlin), 1912, xi, p. 318.

evertsi (in South Africa), Dermacentor reticulatus (in Russia) and Dermacentor nitens (in Panama). It is probable that other species of ticks are capable of disseminating the infection.

Babesia mutans (*Piroplasma mutans*)

Babesia mutans was discovered by Theiler[5] in the blood of South African cattle. The organism is frequently present simultaneously with Babesia bigemina.

Morphology. The parasite is a very small protozoon simulating Theilera parva. It is readily differentiated from the latter organism by the fact that

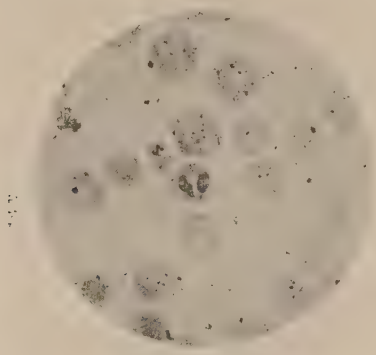


FIG. 73. BABESIA MUTANS

it can be transmitted to normal cattle through the inoculation of blood from infected animals. The organism is found in the form of very small rods, rings or pear-shaped bodies. It occurs in the blood cells singly, in pairs and in groups of three or more.

Babesia mutans is only slightly virulent as compared to Babesia bigemina. It will cause moderate fever, disturbed appetite, loss of weight, and slight anemia. Hemoglobinuria does not occur. In the vast percentage of cases affected animals recover.

Transmission. The transmitting parasite has not been definitely determined. Theiler is of the opinion that Margaropus annulatus is responsible.

[5] Theiler, Rpts. Gov. Bact. Union of South Africa, 1905–1908, incl.

Babesia canis (*Piroplasma canis*)

In certain sections of the world, especially in tropical countries, canine piroplasmosis is a rather common malady. The disease is particularly prevalent in South Africa. It has also been reported from India, China, the Philippines, Italy, France and Russia. The parasite causing the disease was discovered by Piana and Galli-Valerio[6] in Italy in 1895.

The disease, sometimes referred to as "biliary fever" or "malignant jaundice," is characterized by marked anemia, icterus, hemoglobinuria, weakness and emaciation. It proves fatal in a considerable percentage of cases. In a number of instances animals make an apparent recovery but continue to harbor the parasite for long periods of time.

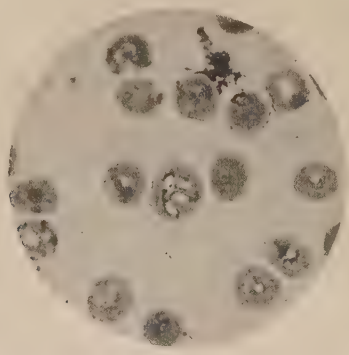


Fig. 74. Babesia canis

Morphology. The fully developed form of Babesia canis is, in general, similar to Babesia bigemina but is somewhat larger. Further, the presence of a large number of parasites in single blood cells is quite common. In some instances a dozen or more organisms will be observed in a single cell. A number of the parasites are frequently found outside of the erythrocytes in the plasma. The organisms present within the cell appear pear-shaped or poly-angular. When present outside of the blood cell Babesia canis is usually round. In fresh preparations the organism can be demonstrated to possess a type of amoeboid movement.

[6] Piana and Galli-Valerio, Il Moderno Zooiatro (Torino), 1895, p. 165.

Cultivation. Thomson and Fantham[7] report the cultivation of Babesia canis by adding 0.10 cc. of a 50 per cent glucose solution to 10 cc. of defibrinated blood containing the parasite and incubating at 37.5°C.

Transmission. Babesia canis is transmitted by Rhipicephalus sanguineus, Haemaphysalis leachi, Ixodes ricinus and Dermacentor reticulatus.

Babesia ovis (*Piroplasma ovis*)

Ovine piroplasmosis was first noted in sheep in Roumania in 1884 by Mazureano who described the malady under the name of "Carceag."

Babesia ovis was first observed in the blood of sick sheep in Roumania in 1892 by Babes,[8] a few years after his findings in bovine piroplasmosis. As in the case of the latter disease, he failed to recognize the true nature of the organism responsible for the malady in sheep. Subsequent investigations revealed the true nature of the parasite.

Besides Roumania, where the disease is of great importance, ovine piroplasmosis (ictero-hematuria, hemoglobinuria) has been reported from Italy, France, Africa, Turkey, China, Germany, Venezuela and the West Indies.

The disease is characterized by fever, inappetence, marked weakness, anemia, icterus and hemoglobinuria. It terminates fatally in a large percentage of cases.

Morphology. Babesia ovis is a little smaller than Babesia bigemina. In the red blood cells it is commonly found as a single, piriform body. Pairs are occasionally observed and irregular forms are not rare.

Transmission. This parasite is transmitted by adult ticks. One species of tick known to transmit it is Rhipicephalus bursa. There are undoubtedly other species capable of disseminating the infection.

GENUS THEILERIA

The genus Theileria was created primarily to separate the causative agent of East African coast fever of cattle from other types of piroplasmata from which it differs in several important respects. The discussion of the type species (Theileria parva) will indicate these differences. At the present time there is no other known species of this genus of importance in veterinary medicine.

[7] Thomson and Fantham, Ann. Trop. Med. and Parsitol., 1913, vii, p. 621.

[8] Babes, Comptes rendus des séances de l'Academie des Science, Paris, 1892, cxv, 359.

Theileria parva

For a time East African Coast fever (Rhodesian red-water, Rhodesian tick fever) was confused with Texas fever. Koch,[9] however, differentiated the malady from Texas fever and Theiler[10] described the etiological agent as a new type of piroplasma. Following this work, various investigators studied the organism and contributed materially to our knowledge of the same.

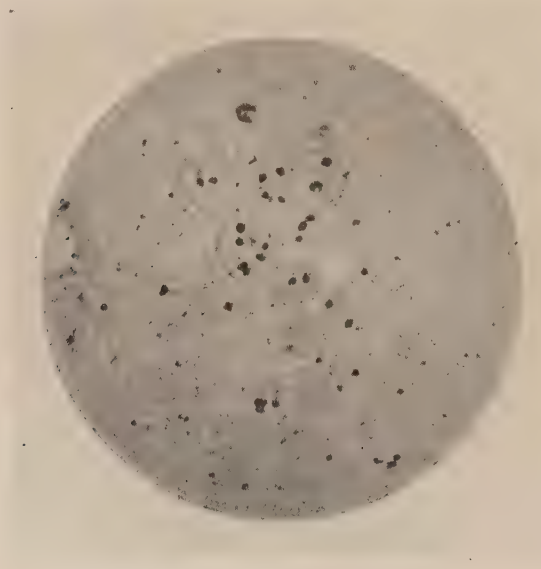


Fig. 75. Theileria parva (in Blood Cells)

Transmission of the disease from affected to normal animals cannot be accomplished through the inoculation of blood containing parasites. However, K. F. Meyer[11] demonstrated that the disease could be transmitted from animal to animal through the inoculation of spleen material.

We are largely indebted to Gonder[12] for a knowledge of the life cycle of Theileria parva.

[9] Koch, Arch. für Wissenschaftliche und Praktische Tierheilkunde, Berlin, 1904, xxx, 281–586.

Koch, Deutsche medizinische Wochenschrift, Berlin, 1905, 1867.

[10] Theiler, Fortschritte der Veterinarhygiene, 1903, i, 133; *ibid.*, 1905, ii, 256.

[11] Meyer, K. F., Jour. Comp. Path. and Therap., London, 1909, xxii, 213.

[12] Gonder, Zeitschrift für Infektionskrankheiten Parasitare Krankheiten und Hygiene der Haustiere, Berlin, 1910, viii, 406.

Morphology. Theileria parva is smaller than any of the piroplasmata. It occurs in the red blood cells, usually in the form of a small, rod-shaped or bacillary body in one end of which is a deep-staining, chromatin granule. While some of the parasites appear as small, pear-shaped structures, large, twin pear-shaped forms are never observed. Ring and disc forms are frequently seen.

About two weeks after infection by ticks there can be demonstrated in the endothelial cells and large lymphocytes of the spleen and lymph glands of cattle the protoplasmic masses first observed by Koch. These contain numerous punctiform or comma-shaped chromatin particles. In addition to their presence in the endothelial cells and lymphocytes, these plasmodial structures are also found extracellular.

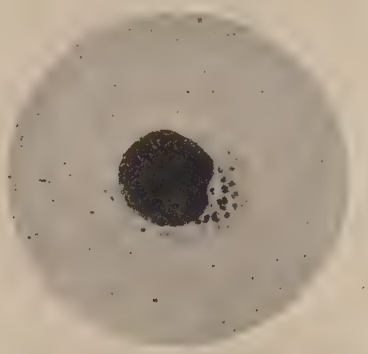


Fig. 76. Koch's Plasma Bodies; Intracellular (Spleen Preparation)

Gonder states that these multinucleated bodies (agamonts) divide into numerous merozoites (agametes). This process is followed by a breaking up of the cell. The liberated merozoites penetrate other cells in the spleen and lymph glands and produce more plasmodial structures. This completes the schizogony process. In course of time merozoites are formed which are capable of multiplying and producing gametocytes. These appear in the red blood cells as microgametocytes (bacillary forms) and macrogametocytes (piriform and round types). When the tick ingests blood containing these gametocytes they develop within the tick's body into microgametes and macrogametes. Copulation occurs and oökinetes are produced. These enter the salivary glands and break up into sporozoites which are passed into a new host by the bite of the tick, thus starting the cycle over again.

Transmission. Theileria parva is transmitted largely by the nymph form of Rhipicephalus appendiculatus. The adult forms of Rhipicephalus simus, Rhipicephalus evertsi and Rhipicephalus nitens are also capable of transmitting the parasite provided they ingested infected blood in their earlier stages of development.

GENUS ANAPLASMA

The genus Anaplasma, created by Theiler, consists of one, or possibly several, species of protozoan organisms which occur as very small, round or oval forms appearing to be made up entirely of chromatin without cytoplasm.

The important member of the genus is Anaplasma marginale. Theiler has recognized another form which he considers a variety of Anaplasma marginale. This second form of parasite differs from the original type in that it is located more centrally in the blood cell, hence it is known as "Anaplasma marginale, variety centrale" or more commonly as "Anaplasma centrale."

Some investigators have doubted that these bodies are protozoan in nature and are inclined to believe that they are degenerative processes. Others are of the opinion that they are forms of Babesia bigemina.

Anaplasma marginale

For a number of years a disease of cattle known as "gall sickness" or "galziekte" and more recently as "Anaplasmosis" has been recognized among cattle in South Africa. The disease is characterized by fever, inappetence, weakness, edema, marked anemia, icterus and emaciation. Thus, it is quite similar to Texas fever with the exception that no hemoglobinuria occurs.

As Theiler points out, in South Africa anaplasmosis commonly occurs simultaneously with Texas fever, hence, it may be confused with such latter disease. Theiler is of the opinion that what appear to be relapses of Texas fever are often cases of anaplasmosis.

Morphology. Anaplasma marginale occurs as a minute, round or oval dot or "marginal point" on the periphery of the red blood cells of animals suffering from anaplasmosis. These bodies measure from 0.1 to 0.5 micron in diameter and usually occur singly in the blood cells although the presence of two parasites in the same cell is not uncommon. As many as 30 to 40 per cent of the red blood cells may be found to contain the parasites.

Transmission. According to Theiler, the organism is transmitted by Margaropus decoloratus and various species of Rhipicephalus.

Anaplasma marginale (*variety centrale*)

This variety of anaplasma differs from the typical "marginale" form in that the parasite is found more towards the center of the red cell. It is also claimed that this variety produces a more mild form of the disease than Anaplasma marginale. Further, it is maintained that animals recovering from the disease due to infection with the "centrale" type of parasite, are not completely immune to infection with the other type.

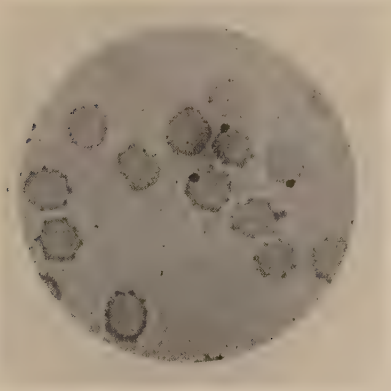


FIG. 77

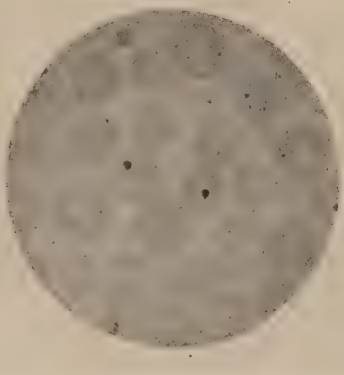


FIG. 78

FIG. 77. ANAPLASMA MARGINALE

FIG. 78. ANAPLASMA MARGINALE (VARIETY CENTRALE)

GENUS EIMERIA (COCCIDIUM)

The characteristics and life history of the parasites of this genus have been discussed in detail in the preceding chapter (see page 346). They may be briefly summarized as follows:

The *coccidial cysts* occur as round or, more often, oval structures, their greatest dimension varying from 15 to 25 microns. These cysts, when mature, contain 4 spindle-shaped *sporocysts* each of which in turn contains 2 sickle-form sporozoites. After the cyst is ingested and opens in the small intestine of the host the liberated sporozoites penetrate the epithelial cells of the intestine and are then known as *trophozoites*. These develop into oval and finally nearly round forms—*schizonts*. Through schizogony a number of spindle-shaped *merozoites* are produced. With the rupture of the schizont the merozoites are liberated and invade new

epithelial cells, becoming trophozoites, then schizonts, and the process of schizogony is repeated. This cycle is repeated a number of times but finally the merozoites, in place of producing more merozoites, develop two types of structures, one, an ovum-like cell known as a *macrogamete* and the other, smaller, spermatozoan-like forms, the *microgametes*. Before maturity the macrogamete is designated a *macrogametocyte*. A form intermediate between the merozoite and microgamete is known as a *microgametocyte*. After the microgametes are liberated they penetrate the macrogamete and fertilize it. This results in the formation of an *oöcyst*. The oöcyst produces 4 *sporoblasts* which develop into sporocysts and these in the mature cyst are found to contain sporozoites. Such cyst when ingested by a host starts the cycle again.

There are two or three species of interest in veterinary medicine Eimeria avium, Eimeria stiedae and Eimeria bovis.

Eimeria avium (*Coccidium avium, Coccidium tenellum*)

Intestinal coccidiosis of domestic fowls is not an uncommon affection, particularly in certain localities. Young birds are more susceptible than older ones.

The disease occurs in an acute, subacute and chronic form, the latter being least common. The malady is characterized by profuse, often bloody diarrhea, depression, weakness, thirst and loss of appetite. Affected birds usually succumb within three or four days. In the subacute cases the course extends over a longer period of time, frequently two or three weeks. The mortality is quite high, particularly among young birds.

Coccidial forms can be demonstrated in the droppings and scrapings from the intestines of dead birds. Cole and Hadley have pointed out that a large percentage of apparently healthy fowls in certain localities show coccidia in their droppings. This fact must be borne in mind in arriving at a diagnosis.

Eimeria avium possesses the basic characteristics of the genus and has no definite distinguishing characteristics.

Eimeria stiedae (*Coccidium cuniculi, Coccidium oviforme*)

Eimeria stiedae is a very common parasite of rabbits. In addition to being found in the intestinal tract it commonly invades the epithelial cells of the bile ducts and liver, bringing about profound changes in the latter organ.

In its characteristics and life cycle Eimeria stiedae does not differ from other members of the genus.

Eimeria bovis (*Coccidium zürni*)

Intestinal coccidiosis or "red dysentery" of cattle has been reported from Switzerland, Denmark, France, Germany and Hungary.

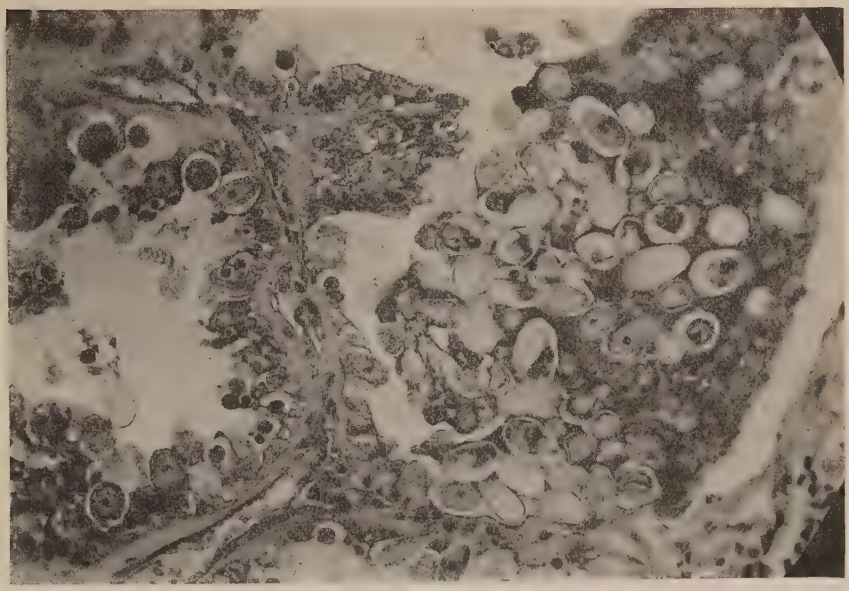


FIG. 79. EIMERIA STIEDAE

Invasion of the epithelium of the bile ducts in the rabbit. Note proliferation of epithelium. Field shows stages in sporogony. Trophozoite stages may be seen in some of the epithelial cells.

The malady affects young cattle principally and is characterized chiefly by a bloody diarrhea. The feces are fetid, contain mucus and frequently rather large clots of blood. In some cases tenesmus is marked and may result in prolapse of the rectum. Protracted cases result in marked weakness, fever, inappetence and emaciation.

The mortality is not very high although a small percentage of affected animals succumb.

Eimeria bovis is similar to other members of the genus as regards its morphology and life cycle.

GENUS ISOSPORA. ISOSPORA BIGEMINA

The oöcysts of the various species of Eimeria are usually discharged from the body of the host with the feces and mature outside. According to Wenyon, in the case of Isospora bigemina the cysts can become fully developed within the host. The main difference, however, between the two genera is found in the formation of the spores. The oöcysts of organisms of the Eimeria genus develop four sporoblasts each of which produces two sporozoites whereas with Isospora two sporoblasts are formed, each producing four sporozoites.

The only known species of Isospora of interest in veterinary medicine is Isospora bigemina. This organism is occasionally found as a parasite of the dog and cat. In place of developing in the superficial epithelium of the intestine Isospora bigemina develops in the submucosa of the intestinal villi.

GENUS SARCOCYSTIS

The parasites of this genus are found as elongated, double-walled capsules commonly known as "Miescher's sacs." In these sacs, which vary in length from 2 to 50 mm. or more, numerous kidney-shaped or crescent-form sporozoites are found. The outer wall of the sac is radially striated while the inner membrane is homogeneous and gives rise to delicate septa which divide the sac into compartments in which the sporozoites are contained.

Sarcosporidiosis is encountered in swine, sheep, cattle, horses, goats, rabbits and various birds. The condition is also occasionally observed in man. The common species found in hogs is *Sarcocystis miescheriana*. In sheep *Sarcocystis tenella* is found. *Sarcocystis bertrami* occurs in the horse. The species commonly found in cattle is *Sarcocystis blanchardi*.

In hogs the abdominal muscles and diaphragm are commonly involved. In sheep the parasites are most frequently observed in the cutaneous and abdominal muscles. In horses the sacs are generally found in the neck and esophageal muscles.

These parasites do not produce disturbances of any consequence except when they are present in very large numbers. The condition, therefore, is not of very great importance.

Very little is known regarding the life history of the organisms of this genus. Some investigators have held the opinion that part of the life cycle is spent in an intermediate host. Some consider that flies play a part in its transmission. The most recent investigations tend to indicate that infection occurs through the alimentary tract.

CHAPTER XXXVII

Infusoria

The Infusoria are highly developed protozoan organisms which are motile by means of relatively short, flexible, vibratile processes known as "*cilia*." By some authors this Class of organisms is designated "Ciliata."

These organisms are more or less oval in outline and possess two nuclei, a *macronucleus* which is concerned in vegetative processes of the organism

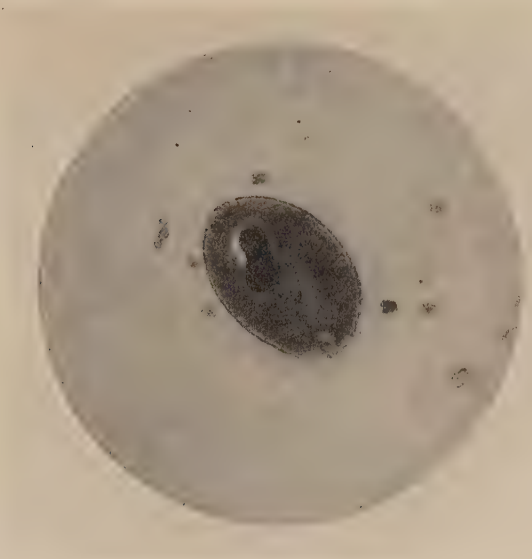


Fig. 80. Balantidium coli

Stained specimen from bowel exudate of Sus philippinensis. (Photomicrograph by United States Army Medical Museum from preparation by Professor Haughwout.)

and a *micronucleus* which plays an active part in reproduction. In some forms the cilia are evenly distributed over the entire cell while with other types they are found distributed all over the cell but, in addition, a particularly prominent, specialized group is found about the mouth orifice (peristome). In still other forms the cilia are not distributed over the body of the cell generally but are found in groups in various locations.

The Infusoria reproduce by simple fission or budding.

While there are a large variety of organisms of this Class found as parasites of the domestic animals there is but one genus which contains species of possible pathogenicity.

The genus referred to is Balantidium and the species Balantidium coli and Balantidium suis. Until rather recently Balantidium coli was the only species recognized as parasitic in hogs. McDonald[1] has distinguished a second species which he designated Balantidium suis.

BALANTIDIUM COLI

While Balantidium coli has been definitely shown to be pathogenic for man, producing a fatal disease in some instances, the organism has generally been regarded as non-pathogenic for the hog. In the latter animal it is a very common inhabitant of the intestinal tract. While it is probably true that this parasite does nor ordinarily produce disease in hogs, Haughwout[2] has demonstrated that, at times at least, it is capable of invading the mucosa and submucosa of the intestine, presenting a microscopic picture similar to that seen in balantidiosis of man (see Fig. 66). It seems highly probable that Balantidium coli is occasionally capable of producing intestinal disturbances in hogs.

For a more detailed discussion of Balantidia, their structure and differential points between Balantidium coli and Balantidium suis the reader is referred to the preceding chapter, page 353.

[1] McDonald, J. Daley, Univ. Calif. Pub. in Zoöl., xx, no. 10, p. 243–300, (1922).
[2] Haughwout, Bull. No. 3, Inst. Med. Res., Fed. Malay States, 1924.

PART VII

THE FILTERABLE VIRUSES

CHAPTER XXXVIII

Disease-producing Viruses Capable of Passing Bacteria-retaining Filters

There is a rather large group of infectious diseases of the lower animals caused by organisms which will pass through clay or porcelain filters (Berkefeld, Chamberland, etc.) which ordinarily hold back the common types of bacteria. With one or two exceptions these so-called *"filterable viruses"* are also ultramicroscopic, i.e., they cannot be seen with the maximum magnification of which our present type of microscope is capable. The virus of contagious pleuro-pneumonia of cattle is one exception. While in watery solutions such virus is capable of passing through Berkefeld and Chamberland filters, with a magnification of about 1500 diameters and a strong light the organism is brought just within the range of visibility.

Another possible exception is the virus of rabies. If, as contended by a number of investigators, the Negri body is a protozoan organism and the cause of the disease, then such stage of the virus is, of course, readily visible microscopically. The filterable stage in such event may be a very minute Negri body, so small that it will pass the filter, or it may be different from the Negri body to which it gives rise.

In the following pages the filterable viruses of importance in veterinary medicine will be discussed.

RABIES VIRUS

Rabies or hydrophobia is one of the oldest diseases known. It is of particular importance because, as is well recognized, the malady is readily transmitted to man as a result of bites inflicted by the lower animals, particularly dogs. Probably no other disease of man is feared or dreaded so much as hydrophobia.

In 1804, Zinke, studying rabies in dogs, demonstrated the infectiousness of the saliva. It was seventy-five years later, however, before any appreciable amount of research was undertaken by laboratory workers. In 1879, Galtier[1] showed that rabies could be transmitted to rabbits through artificial inoculation, and that they developed the disease in the "dumb" or paralytic form. These findings disclosed a convenient and safe method

[1] Galtier, Compt. rend. des seances de l'Acad. des Sci., 1879.

for the experimental study of hydrophobia and marked the beginning of more extensive investigation of the malady.

In 1881, Louis Pasteur[2] with his associates, Chamberland and Roux, found that the virus of rabies had a special affinity for nerve tissue, it being found concentrated in the central nervous sytem. During the next few years (1881 to 1888) the brilliant work of Pasteur,[3] which culminated in the perfection of a successful method of immunization against rabies, was accomplished.

In 1903 Remlinger[4] reported that the virus of rabies could be passed through bacteria-retaining filters and, while microscopically and culturally sterile, would still produce the disease. The following year (1904) Bertarelli and Volpino[5] published a report confirmatory of Remlinger's work. In the United States the filterability of the virus was demonstrated first by Poor and Steinhardt[6] and later by a number of other investigators.

Another very important discovery was made in 1903. During that year Negri,[7] an Italian of Parvia, Italy, described the finding of a certain type of "body" in the cells of the central nervous system, especially in the large ganglion cells of the hippocampus major and in the Purkinje cells of the cerebellum, of animals dead of rabies. These bodies are round or oval and in size vary between wide limits (0.5 to 22 microns). When properly stained, those of appreciable size are found to contain deep-staining granules. Negri believed these bodies to be pathognomonic of rabies and, further, he was inclined to consider them the actual cause of the disease. Confirmation was soon forthcoming as to the presence of these bodies being definitely indicative of hydrophobia. Volpino,[8] D'Amato,[9] Bertarelli,[10] Bosc,[11] Poor[12] and several other investigators published reports in which they indicated that they had likewise observed the bodies described by Negri. Thus, the now well-known method of diagnosing rabies was established, and, in the large percentage of cases, it makes time-consuming, animal-inoculation tests unnecessary.

[2] Pasteur, Comp. rend. d. seances de l' Acad. des Sci., 1881, 1882, 1884, 1885 and 1886.
[3] Pasteur, *Ibid.*, 1881, 1882, 1884, 1885 and 1886.
[4] Remlinger, "Le passage due virus rabique a travers les filtres," Ann. de l'Inst. Pasteur, xvii, p. 834, 1903.
[5] Bertarelli and Volpino, Centralbl. f. Bakteriol., 1te Abt., Orig., xxxvii, p. 51, 1904.
[6] Poor and Steinhardt, Jour. Inf. Dis., xii, p. 202, 1913.
[7] Negri, Zeitschrift für Hygiene und Infektionskrankheiten, 43, p. 507, 1903.
[8] Volpino, Giorn. d. r. Accad. d. med., Torino, 14, p. 228, 1903.
[9] D'Amato, Riforma med., 20, p. 617, 1904.
[10] Bertarelli, Centralbl. f. Bakteriol., 37, p. 556, 1906.
[11] Bosc, Compt. rend. d. l. Soc. d. Biol., 55, p. 1436, 1903.
[12] Poor, Proc. N. Y. Path. Soc., p. 101, 1904.

While the specificity of Negri bodies for a diagnosis of rabies has been established beyond all doubt, investigators have become divided in opinion as to the exact relation which such bodies bear to the disease. In this respect, authorities are largely divided into two groups. One group regards the Negri body merely as a peculiar type of cell degeneration, characteristic of rabies, but of no etiological significance. The second group, with Negri, believe that these bodies are not only diagnostic of rabies, but are protozoan organisms and actually the causative factors.

Those who adhere to the opinion that the Negri body is a process of degeneration, in arguing against its being a definite protozoan organism, point to the filterability of the virus; to the absence of Negri bodies in nerve tissue demonstrated to be infectious, and to the marked scarcity and minute size of the bodies in "fixed-virus" infections, as supporting their contention. On the other hand, those who believe that the Negri body is the inciting factor concerned in rabies, point out that these bodies are exceedingly variable in size and that in some stages of their life cycle it is entirely possible that forms capable of passing bacteria-retaining filters occur. Further, it is argued that these bodies are occasionally seen free, outside of the nerve cells, and that where nerve tissue containing Negri bodies in the cells is triturated and treated with antiformin, microscopic examination will reveal numerous bodies, unaltered in appearance, which have been freed from the cells. Such findings, it is contended, would not be likely were these bodies merely areas of degeneration. Again, their definite outline, regularity of structure and staining characteristics are referred to as opposing the cell degeneration theory.

In 1906 Williams and Lowden[13] pointed out that they had observed cyclic changes in the bodies described by Negri, and concluded that they were a type of sporozoa and the actual cause of the disease. They proposed that they be called "*Neurocytes hydrophobiae.*" Subsequent studies by Calkins[14] support the conclusions of Williams and Lowden.

The author has devoted considerable study to rabies and is inclined to agree with the contention that the Negri body is a protozoan organism and the etiological agent of the disease. In experiments[15] carried out in 1922–1923, a series of rabbits was inoculated subdurally with "fixed virus" and the animals destroyed one at a time after 24-hour periods, commencing 24 hours subsequent to the inoculation. After 72 hours, several days before the animals would have shown symptoms of rabies, structures having all

[13] Williams and Lowden, Proc. N. Y. Path. Soc., 1906; Jour. Inf. Dis., vol. iii, 1906.
[14] Calkins, Proc. N. Y. Path. Soc., 1906.
[15] Kelser, R. A., Jour. A. V. M. A., vol. lxiv, no. 6, 1924.

the characteristics of Negri bodies, and some as large as those found in "street virus" infection, were observed in preparations of the brain tissue undergoing what appeared to be cyclic changes. Some cells were found apparently dividing by fission and in addition swarms of minute, highly refractile bodies, presumably the same as those described by Williams, were seen. In the case of animals destroyed after the appearance of symptoms or which died of the "fixed virus" infection, only a very few, minute Negri bodies were observed. As a result of this work it can be conceived that "fixed virus" consists of ultramicroscopic bodies and those barely visible, and that when these are introduced subdurally in a rabbit they soon attain larger size and undergo cyclic changes. It is believed that this development results in the production of progeny the vast percentage of which are probably ultramicroscopic or nearly so. As a result of the high degree of virulence possessed by virus "fixed" for rabbits the disease runs a brief, acute course, producing death of the animal before the Negri bodies have had time to attain the size observed in the more protracted cases resulting from "street virus" infection.

As might be expected, numerous attempts to cultivate the rabies virus have been made. Nothing of a definite nature has come from such efforts. In 1913 Noguchi[16] published a report in which he indicated that he had been successful in cultivating a protozoan-like organism resembling, in some forms, the Negri body. Up to the present time Noguchi's work has not been duplicated by other investigators.

Diagnosis of Rabies. As indicated above, the demonstration of Negri bodies in brain specimens has been well-established as a definite indication that the animal had rabies. In connection with this method of diagnosis there are several important facts which should be emphasized. First of all, when an animal is killed in the very early stages of rabies Negri bodies of detectable size are frequently absent or are present in such small numbers that they may be missed. If the animal is permitted to die of the disease or is destroyed only after reaching the late stages of the malady, there is no difficulty in demonstrating Negri bodies. If it is destroyed when in the last stages of the disease it should not be shot through the brain. It is obvious that such procedure will result in an unsatisfactory specimen for laboratory examination, yet such method of destroying animals suspected of having rabies is commonly practiced. Another point to be stressed is the proper preparation of the specimen for transmission to the laboratory. If a laboratory doing rabies examinations is

[16] Noguchi, Jour. Exp. Med., vol. xviii, p. 314, 1913.

close at hand and the specimen does not have to be shipped, it is only necessary to remove the head immediately after death and promptly deliver it to the laboratory. If any appreciable delay is to be encountered in getting the head to the laboratory it should be packed in ice. If the specimen is to be expressed or mailed to the laboratory, the most satisfactory procedure is as follows: Carefully remove the entire brain and halve it by a longitudinal cut between the hemispheres. Place one-half in glycerin and the other half in 10 per cent formalin solution, carefully pack and ship to the laboratory. The half preserved in glycerin can be used for "touch" preparations and, if inoculation tests are necessary, such specimen is also suitable for that purpose. If it is desirable to make sections the tissue forwarded in the 10 per cent formalin will be found satisfactory.

In making an examination for rabies the laboratory carries out one or more of three procedures. These consist of the *microscopic examination of "touch" preparations*, *microscopic examination of sections*, and *animal inoculation tests*.

Touch Preparations. If the brain specimen reaches the laboratory in satisfactory condition, the first procedure is to make a microscopic examination of "touch" preparations for Negri bodies. This is accomplished as follows: When the whole head is received, remove the brain and carefully dissect out the hippocampus major (Ammon's horn). If the brain has been forwarded in glycerin and formalin, as outlined above, carefully remove the half of the hippocampus major found in the hemisphere preserved in the glycerin and rinse it lightly in sterile, physiological salt solution. Have ready a number of clean slides, a sharp knife or razor blade, one or two wooden or fibre embedding blocks or a couple of medium-sized corks, and a Coplin jar containing methyl alcohol. With the knife or razor blade cut off a small section of the hippocampus by a clean, transverse cut. Place this section of tissue on the embedding block or cork with the fresh cut surface up. Now touch a slide to the fresh surface of the tissue and by gentle downward pressure flatten it out against the slide. Lift the slide away leaving the bulk of the tissue adhering to the block or cork. The slide will be found to contain a thin film of the nerve tissue. Two or three of such impressions can be made on the same slide which is then immediately placed in the methyl alcohol for fixation of the tissue film. Three or four slides should be thus prepared, using several small segments of the nerve tissue if necessary.

Fixation of the films is complete in about 2 minutes when the slides can be removed and stained. For staining, the author employs Mann's

method for the ordinary examination to detect Negri bodies and Giemsa's method for studying the structure of the bodies.

Where Mann's method is used, the slides containing the fixed films are flooded with freshly prepared stain (see page 92 for preparation of Mann's stain) which is allowed to act 5 to 15 minutes. The slides are then rinsed in water and passed rapidly through 50 per cent, 75 per cent, 95 per cent and absolute alcohol and then cleared in a mixture of equal parts of xylol and cedar oil. In such preparations the nerve cells stain light blue, the Negri bodies a brick red, and the red blood corpuscles an orange color. The Negri bodies are found in the large ganglion cells although they are less often seen in the smaller nerve cells and also extracellular. While Mann's stain gives excellent contrast and for such reason is well suited for ordinary routine diagnostic work, the internal structure of the Negri body is best brought out with Giemsa stain.

In utilizing Giemsa's method best results are obtained when the preparations are permitted to stain overnight. This can be conveniently accomplished by nearly filling a Coplin jar with distilled water and then adding to same 5 or 6 drops of a 1 per cent potassium carbonate solution and 1 drop of Giemsa stain for every cubic centimeter of water. The slides containing the fixed films of nerve tissue are then placed in the jar and left in same overnight. The next morning they are removed, rinsed with distilled water and differentiated in 95 per cent alcohol. The differentiation must be carried out very carefully. The best procedure is to rinse the slide in the 95 per cent alcohol for a few seconds, wash with distilled water, dry and examine with the high power dry lens. If the nerve cells are found to still be a deep purple, the preparation is again exposed for a few seconds to the action of the alcohol, rinsed, dried and examined. Ordinarily this procedure must be repeated several times. By this method, differentiation can be carried to just the desired point where the nerve cells stand out as a pale blue, the nuclei red and the Negri bodies appearing as robin's-egg blue structures containing granules staining purplish-red or azure. For the preparation of Giemsa stain see page 92.

Another satisfactory method of staining Negri bodies is that of Van Gieson's. Williams' modification of this method is very satisfactory. For preparation and use of these stains see page 92.

Sections. When brain specimens reach the laboratory in poor condition so that satisfactory cells cannot be demonstrated, sections are sometimes of value. If part of the brain is received in formalin solution, small sections of the cerebellum and hippocampus are removed, prepared for embedding in the usual way, and sectioned. If the specimen is not received

in formalin and a choice of fixatives is possible, best results are obtained by fixation in Zenker's fluid for about 6 hours.

In staining section Mann's and Giemsa's stains can be utilized. When Zenker's fixation and paraffin embedding are employed, Mallory's eosin-methylene blue method of staining gives good results.

In sections of the cerebellum the Negri bodies are found in the Purkinje cells.

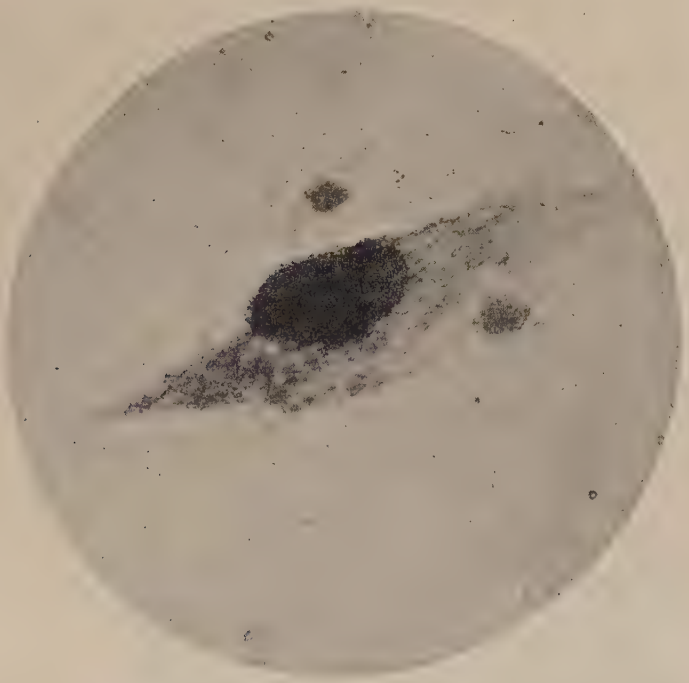


FIG. 81. NEGRI BODY IN GANGLION CELL
(Preparation from hippocampus major; Giemsa stain)

Animal Inoculation Tests. In cases where a brain specimen from an animal suspected of having rabies is received in good condition and satisfactory microscopic preparations (preparations revealing numerous ganglion cells) have been made and carefully studied, the absence of Negri bodies is practically always definite evidence that the animal did not have rabies, provided, however, that the beast was permitted to die or was destroyed

only when about to succumb. If, however, a brain specimen is received in poor condition, making a satisfactory examination impossible, or in cases where the specimen is from an animal destroyed in the early stages of the disease, failure to demonstrate Negri bodies is by no means conclusive evidence of the absence of rabies.

Where persons have been bitten it is good policy to check all negative microscopic examinations with animal inoculation tests. For this purpose either rabbits or guinea pigs can be used. The author prefers rabbits. If the tissue in question is fresh or has been shipped in glycerin, a small amount of same can be well emulsified with sterile physiological saline solution and injected in 0.1 to 0.125 cc. amounts subdurally. If the tissue under examination is in poor condition (decomposing), one of two methods of procedure can be resorted to. One is to place a small amount of the tissue in glycerin for 24 to 48 hours and then prepare it for inoculation. The glycerin will destroy a large percentage of the bacteria but not the rabies virus. The other method and the one which must of necessity be resorted to in some instances, is to thoroughly emulsify some of the tissue in physiological saline solution, centrifugalize it, decant the fluid off of the sediment and filter it through a Berkefeld filter. A small amount of this filtrate is then injected subdurally into several rabbits.

In making subdural inoculations, a small hole can be trephined through the skull and the material injected under the dura mater with a syringe fitted with a small needle. The writer has had excellent results with the following method: A hypodermic needle of 18 gauge is cut off and resharpened so that the needle portion proper measures about $\frac{3}{16}$ inch in length. This is fitted on a tuberculin syringe and a small amount of the tissue emulsion drawn into same. A few centimeters posterior to, and on a line with, the outer canthus of the rabbit's eye, a small horizontal groove can be detected by feeling with the nail of the fore-finger. The hair is shaved from the skin over this area and iodine applied and allowed to dry. With the animal well restrained by an assistant, the needle is placed in the groove mentioned and forced through the bone into the cranial cavity. The bone at this point is thin and can be readily penetrated with very little effort. This method is simple, rapid and satisfactory in every way. The only factor requiring emphasis is the length of the needle. If the needle is too long it will penetrate the brain substance rather deeply and result in a high percentage of losses.

Rabbits inoculated subdurally with brain emulsion containing "street virus" usually develop evidence of rabies within 12 to 16 days. When "fixed virus" is introduced subdurally, the rabbits usually show symptoms in 5 or 6 days and succumb on the seventh or eighth day.

Dissemination of the Infection. Rabies is spread from animal to animal and from animal to man almost entirely through the agency of bites, the saliva of rabid animals being rich in virus. Bites about the face and hands are particularly dangerous. The virus cannot penetrate the unbroken skin and in the absence of lesions in the alimentary canal, infection does not occur through ingestion of the virus.

The saliva of rabid animals is most infectious after the appearance of symptoms. It is, however, infectious to a lesser degree 2 to 5 days previous to the manifestation of symptoms and in a small percentage of cases even 8 to 10 days before evidence of the disease is observed. Thus, if an animal which has bitten another animal or person is kept restrained and under observation for 15 days and at the expiration of such period shows no evidence of rabies it is safe to consider that at the time it inflicted the bite its saliva did not contain rabies virus.

"Street" and "Fixed" Viruses. Rabies virus as found in the saliva or nerve tissue of natural cases of rabies is commonly spoken of as "street virus." Pasteur found that by passing "street virus" successively through a large number (90) of rabbits, its virulence for such species of animal was gradually raised to the point where it would regularly produce symptoms of rabies on the sixth day. After having been raised to such degree of virulence it remains so. Such virus of fixed virulence was designated by Pasteur as "virus fixe."

While rabies fixed virus possesses a high degree of virulence for rabbits when inoculated subdurally, such virus is practically avirulent when administered *subcutaneously* to other animals including man.

Resistance. The virus of rabies is not very resistant. Exposure to air and sunlight destroys it rapidly. Drying in the presence of air likewise renders it inert in a short time. A temperature of 58°C. destroys it in one-half hour. Boiling is immediately effective. Phenol in 5 per cent solution kills it in one-half hour; 1:1000 bichloride of mercury in 15 minutes. Liquor cresolis compound in 3 per cent solution and 1 per cent formalin destroy it in 15 minutes. The rabies virus will resist putrefaction for a long period of time if protected from other detrimental influences. Glycerin will only destroy the virus of rabies after weeks of exposure. If the brain tissue is cut up in minute pieces or emulsified it loses its virulence in glycerin after several weeks. When larger pieces of tissue are preserved in glycerin the virus in same remains active for months.

Immunization. One of Pasteur's outstanding accomplishments was the development of the now well-known Pasteur method of immunization against rabies. He found that the virus contained within the spinal

cords of rabbits dead of fixed virus rabies, could be gradually reduced in virulence by drying the cords over sodium hydroxide. Thus, he found that an emulsion of a cord dried for five days would, after subdural inoculation, require 8 days to produce rabies in a rabbit. When the cord was dried 9 days it required 15 days to produce the disease and when dried for two weeks it was found to be completely avirulent. Putting this finding to practical use Pasteur developed his system of vaccination.

He started by giving a subcutaneous injection of material prepared from the most attenuated cord and followed this with subsequent injections of less attenuated material, gradually working up to the point where a preparation of the least attenuated cord was administered.

As now commonly carried out by a number of institutions, the "Pasteur treatment" extends over a period of 21 to 25 days and consists of the injection of emulsions of cords dried for periods varying from 8 to 2 days. In some of the so-called "intensive treatments" given to individuals badly bitten, especially about the face or hands, several of the injections in the course of treatment consist of preparations of cords dried only one day.

Pasteur's method of immunization, or some modification of the same, has been employed extensively throughout the world and has proven of tremendous value in protecting exposed individuals from the dreaded malady. Lower animals are likewise afforded protection by such method of immunization, but up until recently the treatment of animals has been practiced only on a limited scale, largely because of the cost of treatment.

Several other methods of immunization against rabies have been developed. The method of Högyes has been employed on a rather large scale. In this method dilutions of fixed virus are administered, starting with a high dilution and gradually increasing the amount of virus given. As previously indicated it has been shown that fixed virus can be administered *subcutaneously* to man, dogs, horses, cattle, etc. without ill effect.

Another method was developed by Harris. He froze the virus cord and brain with carbon dioxide snow, finely ground up the same while in a frozen state and dried the material over sulphuric acid in a vacuum desiccator kept at a temperature of $-18°C$. After drying, the powder is put up in sealed tubes *in vacuo* and the same stored at a temperature below the freezing point. This method eliminates drying cords for different periods of time and large amounts of the product can be prepared at one operation, as it is claimed that the material can be stored for at least 6 months without further loss of virulence. The original process reduces the virulence of the fixed virus about 50 per cent. When ready for use, a definite

amount of the powder is weighed out and diluted with physiological salt solution, the amount of powder administered being gradually increased as the treatment progresses. The Harris method has not come into use on a very large scale.

Cumming has shown that an emulsion of virus cord and brain can be placed in collodion sacs and dialyzed against distilled water with the result that the material remaining in the sac loses its ability to produce rabies when inoculated subdurally in rabbits but when employed as a vaccine will effectively prevent the disease. Vaccine prepared by this method has apparently given very satisfactory results. This type of vaccine will have to be more generally used before it can be accurately compared with the Pasteur and Högyes methods.

During the last few years the veterinary profession has paid considerable attention to the so-called "single injection method" of immunizing dogs against rabies. This method was developed by Umeno and Doi[17] in Japan and consists of the injection of an emulsion of fixed virus tissue to which phenol and glycerin has been added, the phenol destroying the virus or at least so attenuating it that it is incapable of producing rabies when inoculated subdurally into rabbits. This method was first introduced into the United States by Eichhorn and is being used on a fairly large scale.

Experiments conducted by Schoening,[18] of the United States Bureau of Animal industry, tend to indicate that while the "single injection method" will immunize effectively against some strains of street virus it will not produce immunity against all strains. Schoening is of the opinion that failure to protect against certain strains of street virus is possibly due to the existence of more than one "type" of street virus in the United States. As the strain of fixed virus used by the different institutions in the United States producing rabies vaccine for the "single injection method" was found to be from the same source it appears possible that the vaccine from such strain might not protect against all "types" of street virus. The author is more inclined to believe that the difference between the strains of street virus will be found to be variations in virulence and not separate and distinct types of virus.

For the details of preparing the various vaccines for immunization against rabies the reader is referred to the section on the Preparation of Veterinary Biological Products.

Antirabic Serum. An antirabic serum prepared by immunizing sheep

[17] Umeno and Doi, Kitasato Arch. Exper. Med., vol. iv, no. 2, March, 1921.

[18] Schoening, Jour. Agric. Research, vol. xxx, p. 431, March, 1925.

by a series of intravenous injections of fixed virus has been used in the treatment of rabies. The results have been disappointing.

VIRUS OF BOVINE CONTAGIOUS PLEURO-PNEUMONIA (ASTEROCOCCUS MYCOIDES)

Contagious pleuro-pneumonia or "lung plague" of cattle is an important disease in certain sections of the world. Fortunately, the malady does not exist in the United States at the present time. It was prevalent in this country, however, during the latter part of the last century and caused the death of thousands of animals, particularly in Illinois. Through energetic measures the disease was finally eradicated in 1892 and since that time has not made its appearance in the United States.

In 1898 Nocard and Roux discovered the organism responsible for the disease and succeeded in cultivating it. This organism is so minute that it will pass through Berkefeld and Chamberland filters except when contained in undiluted albuminous exudates. With a magnification of at least 1500 diameters the organisms appear as minute, highly refractile, motile "points" or dots. Careful study will show that the virus is quite pleomorphic for besides the small dots or coccoid bodies, cultures are found to contain minute spiral forms, asteroid figures and myceloid structures. Because of this morphology, Borrel designated the organism "Asterococcus mycoides."

The virus of contagious pleuro-pneumonia is found in concentrated form in pulmonary and pleural exudate. It is also present in the lymph glands of the thoracic cavity, in the bronchial secretions and in the nasal discharge. Only rarely can it be demonstrated in the circulating blood.

Under natural conditions the infection appears to be spread by inhalation of air contaminated with material from the respiratory tract of affected animals. Direct contact between healthy and affected animals is essential. There is no evidence that the disease can be transmitted through the ingestion of infectious material.

Originally the virus of contagious pleuro-pneumonia was cultivated by inoculating a small quantity of exudate, removed with aseptic precautions from affected tissue, into collodion sacs containing bouillon and placing these sacs in the abdominal cavity of a rabbit. After 15 to 20 days the sacs were removed and the fluid contained in same was found to be slightly turbid. Microscopic examination, with high magnification, revealed the organism already described. Such cultures, inoculated into cattle, readily produced the disease.

The virus of contagious pleuro-pneumonia can be cultivated outside of

the body in Martin's bouillon to which about 6 per cent beef or rabbit serum is added. Cultures in such medium incubated 48 to 96 hours at 37.5°C. will show a slight turbidity resulting from the propagation of the organism. The virus can also be grown on solid medium prepared by adding agar or gelatin to Martin's bouillon. When a few drops of exudate or fluid culture are placed upon such medium and incubated at 37.5°C. for 3 or 4 days extremely minute, transparent colonies can be discerned by the aid of a lens. These colonies later become more dense, appearing as minute, whitish points which adhere tenaciously to the medium.

Resistance. The virus contained in the pulmonary exudate loses its virulence rapidly when exposed to air and sunlight. It is destroyed within 20 minutes by a temperature of 60°C. When kept at temperatures below the freezing point the virus will survive for several months. Glycerin is ineffective and of the ordinary chemical disinfectants phenol is the least satisfactory as the virus will resist 0.50 per cent phenol for a considerable period of time.

Cultures lose their virulence in a few days unless hermetically sealed and kept at low temperature.

Immunization. Vaccination with pleural exudate from cases of contagious pleuro-pneumonia has been practiced. This material is injected subcutaneously in the tip of the tail. This method has not been entirely satisfactory as from 1 to 5 per cent of the vaccinated animals die and as many as 15 per cent may lose their tails.

Vaccination with culture preparations has been carried out, particularly in France, with somewhat more satisfactory results. In this method cattle are given 0.125 to 0.5 cc. of an eight-day old culture in the tip of the tail. However, even with this method, a small percentage of losses occurs and quite a number of animals lose their tails.

An anti-serum, possessing some merits, can be prepared by administering increasing amounts of culture subcutaneously to recovered or vaccinated cattle.

HOG CHOLERA VIRUS

Hog cholera is the greatest menace of hogs the livestock industry has to contend with. The disease is widespread in North America, France, Germany, England and, in fact, occurs in practically all European countries. The disease was introduced into the United States in 1833 in the State of Ohio. Since that time it has spread over the entire country and annually causes great losses.

In 1904 de Schweinitz and Dorset[19] found that the blood of hogs suffering from a disease clinically indistinguishable from hog cholera, when filtered through bacteria-retaining filters and inoculated into healthy hogs, reproduced the disease. This important finding was immediately followed by other experiments by Dorset, Bolton and McBryde[20] in which they conclusively showed that true hog cholera is caused by an ultramicroscopic, filterable virus.

Up until the time of de Schweinitz and Dorset's report it was considered that Salmonella suipestifer, discovered in 1885 by Salmon and Smith, was the cause of hog cholera. We now know that this organism is only a secondary factor.

Numerous attempts have been made to bring the virus within the range of visibility and a still greater amount of work has been done with a view to cultivating the agent. All such efforts have failed.

The virus of hog cholera is highly infectious for susceptible animals. In affected hogs it is found in the blood in concentrated form, especially at the height of the disease. The urine of animals suffering from the malady is very infectious and is the chief agent in the dissemination of the disease. The ordinary mode of infection is through the ingestion of contaminated food and water.

Resistance. A temperature of 65°C. will destroy the virus of hog cholera in one hour when present in blood or fluids. In a dry state a temperature of 75°C. is required. The virus is quite resistant to phenol and glycerin and also bichloride of mercury in the presence of albuminous material. Formalin is an effective agent for its destruction.

Immunization. The greatest single weapon we possess for the control of hog cholera is an effective method of immunization. For the development of this method we are indebted largely to Dorset, McBryde and Niles. Recovered or immunized hogs are hyperimmunized by injecting them intravenously with virulent blood. Such animals are subsequently bled and their blood (defribinated or only the serum) used to immunize susceptible animals and treat those already showing symptoms of the disease.

For prophylactic purposes two methods are in common use. One is known as the "serum alone method" and consists merely in the administration of blood or serum from a hyperimmunized animal. The immunity conferred by such method is of very short duration (several weeks). The

[19] de Schweinitz and Dorset, Rpt. of U. S. Bureau of Animal Industry, xx, for 1903, p. 157.

[20] Dorset, Bolton and McBryde, Rpt. of U. S. Bureau of Animal Industry, xxi, for 1904, p. 138.

other method is known as the "simultaneous method" and consists of the simultaneous administration of immune serum and virulent blood. When used under proper conditions by a competent veterinarian, the latter method confers a lasting immunity and is not attended by any appreciable danger. In the hands of laymen or incompetent individuals many losses frequently follow the use of virus.

For details in connection with the preparation of anti-hog-cholera serum see the section on the Preparation of Veterinary Biological Products.

EQUINE INFLUENZA VIRUS

In veterinary practice the general term "shipping fever," covers collectively that group of epizootic infections which are commonly incident to the shipment of horses and mules. In this group we recognize three separate and distinct entities, namely, influenza, contagious pneumonia and strangles.

In influenza proper, we recognize an acute, febrile, systemic infection, characterized chiefly by catarrhal inflammation of the mucous membranes, and accompanied frequently with inflammatory swellings of the tendons, tendon-sheaths and subcutis.

Contagious pneumonia is that condition which has frequently been spoken of as the pectoral form of influenza. It manifests itself as a croupous or pleuro-pneumonia. That contagious pneumonia is a separate and distinct entity, apart from influenza, is now more or less generally conceded by most investigators. This view is substantiated by the fact that a type of filterable virus has been proven the cause of influenza, whereas no such evidence is forthcoming in contagious pneumonia; and further, by the fact that animals recovering from true influenza possess a substantial immunity against further attacks of that disease but are still readily susceptible to contagious pneumonia. Conversely, horses which have suffered from contagious pneumonia are less susceptible to further attacks of such disease but are readily susceptible to influenza. Of course, it should be recognized that influenza and contagious pneumonia run hand in hand. Factors promoting one disease are equally favorable for the other. Thus, the two diseases often occur simultaneously in the same group or stable of animals.

In 1882, Dieckerhoff demonstrated that influenza could be transmitted to susceptible animals by the intravenous and subcutaneous injection of blood from affected animals. Later Poels demonstrated the filterability of the virus by producing the disease in susceptible horses through the inoculation of Berkefeld-filtered semen from a stallion which was known

to have infected mares which it had been permitted to serve. Subsequently, Poels produced the disease by the inoculation of Berkefeld-filtered blood-serum from a horse which had been artificially infected. Bacteriological examination of these virulent, filtered inoculums failed to give positive cultural results or to reveal visible organisms microscopically. Thus, the causative factor in equine influenza has been established as an ultramicroscopic, filterable virus. These results have been confirmed by a number of investigators.

Transmission. The exact manner in which the influenza virus is transmitted from animal to animal is not definitely known. It is probable that the common mode of entrance of the virus into the body is by way of the alimentary tract. Infection through the respiratory tract, while not proven, may possibly occur. Secondary influences which lower the natural resistance of an animal play a tremendous part in the incidence of influenza. We recognize that conditions incident to shipment are potent contributory factors. Energetic sanitary measures, coupled with such means as can be adopted to protect the animal against conditions which tend to lower its normal resistance, constitute the best we have to combat the disease.

Immunization. Numerous types of biological products have been prepared and marketed for the prevention and treatment of equine influenza. Bacterins and vaccines prepared from organisms found as secondary invaders, have been used on a rather large scale. Serum has been prepared by immunizing horses against the secondary bacteria and, in some cases, by giving horses which have recovered from influenza subsequent injections of blood from animals in the acute stages of the disease. Unfortunately the results obtained with these various products have been discouraging.

EQUINE INFECTIOUS ANEMIA VIRUS

Equine infectious anemia or "swamp fever" occurs as an acute or chronic, septicemic affection, characterized by fever, depression, marked weakness, edema and a profound anemia due to the destruction of a large percentage of red blood cells. In the acute cases the disease ordinarily extends over a course varying from 4 or 5 days to 2 or 3 weeks. The chronic type runs a course of from one to several months or may even extend over a period of years. Schalk and Roderick[21] reported a case which extended over 14 years. At periods during this time the animal appeared perfectly well but its blood was shown to be constantly infectious.

[21] Schalk and Roderick, N. Dak. Agric. Exp. Sta. Bull. 168, August, 1923.

In North America the disease occurs in Canada and the United States. In the latter country it has been reported from Minnesota, North Dakota, South Dakota, Nebraska, Wyoming, Montana, Nevada, Kansas, Michigan, New York, Virginia and Texas. It is highly probable that it occurs in other states.

While the infectious character of the disease was established in 1859 by Anginiard, the nature of the causative agent was not discovered until about 45 years later. In 1906 Carré and Vallée[22] reported the etiological factor of infectious anemia as an ultramicroscopic, filterable virus. The following year (1907) Ostertag[23] published a report confirmatory of Carré and Vallée's finding. Since then the fact that the disease is due to a filterable virus has become well established.

The virus of equine infectious anemia is continually present in the blood of affected animals and is eliminated with the urine. Some writers indicate that the feces are also infectious. Such is the case only when the fecal matter is contaminated with urine.

The disease is spread from animal to animal through the ingestion of food and water contaminated with urine from affected animals or carriers. The possibility also exists that the infection is spread by biting flies.

Animals which have made an apparent recovery from the disease are constant carriers of the virus, the same being present in the blood stream and is eliminated with the urine. Such carriers suffer relapses and while the affection may extend over a period of years, in practically all cases the animal will finally die of the disease.

Resistance. The virus of equine infectious anemia is destroyed within 15 minutes by a temperature of 58°C. The ordinary chemical disinfectants in the usual strength solutions render the virus inert within a short period of time. On the other hand, the virus resists putrefactive changes and drying for several months.

Immunization. The disease eventually terminates fatally in practically 100 per cent of cases. Numerous instances are on record where animals have made temporary recoveries, apparently gaining considerable tolerance for the virus. In such cases animals frequently gain weight, develop an increase in blood cells, etc. Relapses occur, however, and death finally results.

Up to the present time no method for the immunization of horses against infectious anemia has been developed.

[22] Carré and Vallée, Revue générale de médecine vétérinaire (Toulouse), 1906, viii, 593.

[23] Ostertag, Zeitschrift für Infektionskrankheiten, Parasitäre Krankheiten and Hygiene der Haustiere (Berlin), 1907, iii, 1.

VIRUS OF APHTHOUS FEVER (FOOT-AND-MOUTH DISEASE)

Aphthous fever or, as it is more commonly known, foot-and-mouth disease, of cloven-footed animals, caused by an ultramicroscopic, filterable virus, is one of the most contagious diseases known to the veterinary profession. It occurs in various parts of Europe, Asia, Africa and South America.

Of the important infectious diseases of animals ordinarily foreign to the United States, aphthous fever has been introduced more often than any other. A half dozen outbreaks have been experienced during the last 56 years. Several of these outbreaks, particularly the one of 1914–1915, were of a very serious nature. However, through the energetic and efficient efforts of the Federal Bureau of Animal Industry and State livestock authorities the malady has each time been effectively eradicated.

The filterability of the virus of aphthous fever was first established in 1897 by Loeffler and Frosch.[24] This finding was soon confirmed by other investigators and at the present time is a well-established fact.

Frosch and Dahmen recently reported the identification and cultivation of what they considered to be the virus of aphthous fever. They designated the organism, which they believed they had discovered with the aid of an ultramicroscope (a microscopic process involving violet ray illumination and photography), "Loffleria nevermanii." Other investigators have failed to confirm this report.

Aphthous fever manifests itself as an acute, febrile affection, during the course of which characteristic vesicles develop on the mucous membranes, particularly of the mouth, and on the skin of the interdigital spaces, udder, etc. The disease occurs in cattle, hogs, sheep, goats, deer and other cloven-footed animals. Man is also susceptible. In the human family the disease usually runs a very mild course, especially in adults. In young children, however, it occasionally assumes a serious character. The ordinary laboratory animals are not susceptible to the disease.

The virus of aphthous fever is contained in concentrated form in the fluid of the vesicles which characterize the disease. As those present in the mouth rupture, their contents contaminate the saliva and this in turn infects food, water, pastures, etc., and is a potent factor in the spread of the infection. Vesicles on other parts of the body, of course, discharge infectious material which likewise contaminates various objects. In the

[24] Loeffler and Frosch, Centrablatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, Originale, (Berlin), 1897, xxii, 257.

initial, febrile stages of the disease the virus is present, to a limited extent, in the blood.

Resistance. When maintained at a temperature ranging between −4° and 2.5°C. the virus of aphthous fever remains virulent for four months or more. When kept at temperatures ranging between 3.5° and 5.5°C. it will retain its virulence for six months or longer. Heating at a temperature of 55°C. destroys the virus in 15 minutes. When dried in the air and sunlight it loses its virulence in 24 hours. Bichloride of mercury (1:1000), 5 per cent phenol, 3.5 per cent liquor cresolis compound and 1 to 2 per cent formalin kills the virus within one hour. On the other hand, the virus of aphthous fever is very resistant to a number of chemicals. It will tolerate the action of 50 to 60 per cent ethyl alcohol for 16 to 18 hours and is not destroyed in a saturated solution of chloroform in 36 hours.

Immunization. Several methods of immunization have been advocated but up to the present time no method can be considered sufficiently satisfactory to warrant its general use.

CANINE DISTEMPER VIRUS

As indicated in the discussion of Alcaligines bronchisepticus, there has been considerable debate during the last ten years as to whether canine distemper is due to Alcaligines bronchisepticus or to a filterable agent other than the organism named. While it cannot be said that the matter has been settled beyond all doubt, the writer adheres to the belief that the primary etiological factor of the disease is an ultramicroscopic, filterable virus and that Alcaligines bronchisepticus is present as a secondary invader.

In 1905 Carré[25] reported the results of investigations which indicated that the causative agent of canine distemper belonged to the group of ultramicroscopic, filterable viruses. This finding has been confirmed by a number of investigators.

Distemper is a very common and highly important disease of young dogs. It occurs to a lesser extent among cats and other young, carnivorous animals. The affection is characterized by fever, acute catarrah of the mucous membranes, especially those of the respiratory tract, and often results in a catarrhal pneumonia. In some instances the disease involves the nervous system giving rise to marked nervousness, spasms of groups of muscles, convulsions, paralysis, etc.

The distemper virus is present in the nasal secretion in the early stages of the disease. It is also present in the circulation at the onset of the affec-

[25] Carré, Bulletin de la Société centr. de médecine vetérinaire (Paris), 1905, 335.

tion. Infection occurs through the ingestion of food and water contaminated with the virus and, in addition, there is evidence tending to indicate that it may occur by way of the respiratory tract.

Resistance. The virus of canine distemper is destroyed within 20 minutes by a temperature of 58°C. It also succumbs after rather short exposure to the common antiseptics in the usual strength solutions.

Immunization. Various vaccines, bacterins, serums, etc., have been prepared and marketed for the treatment and prevention of canine distemper. The results generally have not been satisfactory.

RINDERPEST VIRUS

Rinderpest or "cattle plague" is a highly fatal disease of the bovine species in various parts of Asia, Africa and Europe. The disease does not exist in the United States proper, but in the Philippine Islands, at the present time, it is by far the most important livestock problem the Insular authorities have to deal with.

Rinderpest is an acute, febrile affection characterized by a croupous, diphtheritic inflammation of the mucous membranes of the alimentary tract. A pronounced stomatitis, marked by erosions of the mucous membranes of the mouth, occurs. The inflammation of the digestive tract is particularly evident in the fourth stomach, region of the ileo-cecal valve, and rectum. A profuse, usually bloody, diarrhea occurs.

In 1902 Nicolle and Adil-Bey[26] published the results of investigations which definitely indicated that the etiological factor of rinderpest belonged to the ultramicroscopic, filterable viruses. This finding has since been well substantiated by the researches of other investigators.

In animals suffering from rinderpest the virus can be demonstrated in practically all the body tissues and fluids. Thus, the urine, feces, saliva, vaginal discharge, nasal secretions, etc., of an affected animal are all infectious. The virus is present in the blood in great amounts at the height of the disease and appears to be adherent to the red blood cells. If such cells are removed from infectious blood, without laking, the remaining clear serum will be found to possess little or no virulence.

Rinderpest virus is passed from animal to animal largely through the agency of contaminated food and water.

Resistance. Kept in a fluid state protected from air, light, and high temperatures the virus of rinderpest will survive several months without difficulty. Drying in the air and sunlight renders it inert in several days.

[26] Nicolle and Adil-Bey, Annales de l'Institute Pasteur, xvi, 1902, 56.

Bile will destroy it in 2 or 3 hours. A temperature of 60°C. destroys the virus within 5 minutes. Of the chemical disinfectants 1:1000 bichloride of mercury, 5 per cent phenol, 3.5 per cent liquor cresolis compound, etc., are effective agents for the destruction of rinderpest virus. Phenol in weak dilutions (0.5 per cent) is ineffective. Glycerin has been emphasized as an effective agent in destroying rinderpest virus. The author has recently had considerable experience with glycerin as a germicidal agent for this virus and finds that while it will destroy it when present in fluids free of suspended material, it will not destroy it if contained in tissue particles in suspension. Suspensions of minute particles of virulent tissue in blood containing $33\frac{1}{3}$ per cent glycerin have been found virulent for a month or more.

Immunization. Several methods of immunization against rinderpest have been developed. The method which has been employed on the largest scale is the simultaneous method of Kolle and Turner. In this method susceptible animals are given 10 to 25 cc. of immune serum subcutaneously in one place and 1 cc. of virulent blood in another part of the body. There are several objections to the simultaneous method. First of all, a certain percentage of losses occur as a direct result of vaccination. Then, in most localities where rinderpest has been particularly prevalent bovine piroplasmosis and trypanosomiasis also happen to be common so the possibility exists that such infections may be spread to animals receiving virus from cattle suffering from rinderpest and also carrying piroplasmata or trypanosomes. Finally, the simultaneous method frequently results in the spread of rinderpest to unvaccinated animals which may come in contact with those recently vaccinated.

The serum alone confers a passive immunity of short duration and is also of value in the treatment of the disease if used early and given in large amounts.

Boynton, working in the Philippine Islands, has recently developed a vaccine for immunization against rinderpest. This product is at present being used on a rather large scale in connection with the control of rinderpest in the Philippines. The details of this work have not as yet been published.

The writer has prepared a rinderpest vaccine consisting of an extract of tissues from affected animals, the virus in such product being killed through the use of chloroform. To date highly satisfactory results have been obtained with this vaccine. The details of this work will shortly be published.

VIRUS OF AFRICAN HORSE SICKNESS

For over a century there has been recognized in South Africa a disease of the equine species known as "African horse sickness" or "pestis equorum." This malady runs a fatal course in most cases and has been responsible for great losses in the horse and mule population of southern Africa.

The disease is characterized by a gelatinous infiltration of the subcutaneous and intramuscular tissues, particularly of the ocular and throat regions, catarrhal inflammation of the stomach and duodenum, acute swelling of the superficial lymph glands, pulmonary edema and hemorrhages in the internal organs. It occurs usually during warm months in the rainy season. After the frosts occur the spread of the disease stops. It is also a curious fact that the infection is readily spread at night whereas horses pastured on infected premises during the day time are not affected.

In 1900 McFadyean[27] reported that the virus of African horse sickness was capable of passing a porcelain filter. The following year (1901) Nocard[28] confirmed McFadyean's work and since that time a number of investigators have conducted investigations in which the filterability of virus has been fully established.

The virus is present in the blood during all stages of the disease. It also occurs in the body exudates and bronchial secretions but has not been demonstrated in the excrements. Unlike infectious anemia, recovered animals do not harbor the virus in their blood.

The disease can be readily transmitted to a susceptible animal through the subcutaneous or intravenous injection of minute amounts of blood from an infected animal. On the other hand, if virulent blood is administered to a susceptible animal by mouth it requires large amounts (150 cc.) to produce the disease. This latter fact, together with the knowledge that infection usually occurs at night, seems to indicate that some intermediate agent which is active at night is probably responsible. In this connection mosquitoes have been suspected.

Resistance. The virus of African horse sickness will resist putrefaction, temperatures as high as 45°C., weak phenol solutions, etc. It is destroyed within 20 minutes by a temperature of 60°C.

Immunization. Several methods of immunization have been employed but the one probably giving best results is that advocated by Theiler who has devoted considerable study to the disease. In this method suscepti-

[27] McFadyean, Jour. Comp. Path., 1900, xiii, 1.

[28] Nocard, Bulletin de la Société Centr. de Médecine Vétérinaire, (Paris), 1901, 37.

ble animals are given, intravenously, a 300 cc. dose of serum from hyperimmune animals. This is immediately followed by a subcutaneous injection of 1 to 2 cc. of virulent blood. The temperatures of the vaccinated animals are taken several times daily and upon the appearance of a febrile reaction a second injection of 50 to 100 cc. of serum is administered. While a small percentage of losses occur with this method, the results, in general, are satisfactory.

In preparing the serum Theiler recommends the use of various strains of virus as it appears that serum prepared from one strain only, will not afford effective protection against infection with all strains.

FOWL PEST VIRUS

Under the name of "fowl pest" or "fowl plague" we recognize an acute contagious disease of fowls, particularly chickens and turkeys, which is characterized by hemorrhages in various tissues of the body and in some cases edematous infiltrations. Petechial hemorrhages or ecchymoses are found in the proventriculus, gizzard, serous membranes, adipose tissue, intestines, etc. In many respects the disease resembles fowl cholera and for years was apparently mistaken for same.

Fowl pest, as a specific disease, was first studied and described by Perroncito[29] in Italy in 1878. The disease was subsequently recognized in various other European countries, particularly Germany, France and Belgium. In the western hemisphere it has been reported from Argentina, Brazil and in the latter part of 1924 it made its appearance for the first time in the United States.

The nature of the virus was first discovered by Centanni and Savonuzzi.[30] In 1901 these investigators reported that the virus was capable of passing a porcelain filter, thus establishing its place in the group of ultramicroscopic, filterable viruses. This finding was soon confirmed by other workers and at present is a well-established fact.

The virus of fowl pest is present in the blood, in the body exudates, droppings and nasal secretions. The disease is spread chiefly through the ingestion of contaminated food and water. As small an amount as one millionth of a cubic centimeter of virulent blood injected into a susceptible chicken will produce the disease. It is reported that geese, ducks and pigeons are susceptible to the disease. While the disease has been ap-

[29] Perroncito, Quoted from Freese in Deutsche tierärztliche Wochenschrift (Hanover) 1908, 173.

[30] Centanni and Savonuzzi, Quoted from Freese in Deutsche tierärztliche Wochenschrift (Hanover), 1908, 173.

parently observed in such species in European outbreaks, the strain of virus responsible for the outbreak in the United States was highly virulent for chickens, somewhat less so for turkeys and practically avirulent for ducks, geese and pigeons.

Resistance. The virus of fowl pest is destroyed within five minutes by a temperature of 65°C. Direct sunlight destroys it within a very short time. When protected from air and sunlight and kept in a cool place the virus will remain active for several months. Bichloride of mercury 1:1000, 5 per cent phenol, 3.5 per cent liquor cresolis compound and 2 per cent formalin destroy it within one-half hour.

Immunization. No satisfactory method of immunizing birds against fowl pest has been developed.

VIRUS OF FOWL POX AND AVIAN DIPHTHERIA

Evidence accumulated during recent years, indicates that avian diphtheria and chicken or fowl pox, which have been considered separate and distinct diseases, are but different manifestations of infection with a single virus. While several investigators previously expressed the opinion that the virus of avian diphtheria and that of chicken-pox were identical, it remained for Carnwath,[31] in 1908, to bring forth experimental evidence in support of such contention. This investigator was able to produce avian diphtheria with pure pox material and chicken-pox with material from avian diphtheria cases. While these findings are held as inconclusive by a few authorities, they have been confirmed by others and at the present time it can be considered fairly well established that avian diphtheria and chicken-pox are caused by the same etiological agent.

The diphtheritic condition is characterized by the formation of croupous or pseudo-membranes on the mucosa of the mouth, throat, nasal cavity, conjunctivae, etc. The tissues in the orbital region frequently become swollen to such an extent that the eye is completely closed. In the pox form, nodular eruptions develop on various parts of the skin, particularly on the comb, wattles, around the eyes and on other parts of the body where feathers are absent or scarce. The two forms of the disease are frequently observed in the same bird.

The malady occurs most frequently in chickens but turkeys, pigeons and other birds are susceptible. Water fowl are fairly resistant. The disease occurs in various sections of the United States and is also prevalent in Europe and Asia.

[31] Carnwath, Arbeiten aus dem Kaiserlichen Gesundheitsamte (Berlin), xxvii, 388.

In 1902 Marx and Sticker[32] demonstrated the virus of chicken-pox to be capable of passing a porcelain filter. This finding was soon confirmed by other investigators and at the present time is a well-established fact.

In connection with avian diphtheria and pox, minute organisms known as "chlamydozoa," have been demonstrated in emulsions of material from lesions. These are found free and also in the epithelial cells. They are considered by some authorities as the carriers of the filterable virus.

The virus of avian diphtheria and pox is present in the epithelial nodules and diphtheritic material. In the initial stages of the disease it is present in the blood but only for a brief period of time.

Resistance. This virus is quite resistant. It will survive drying for a period of several weeks. It will resist weak dilutions of phenol for a considerable period of time and will live in pure glycerin for 2 to 3 weeks. A temperature of 65°C. destroys it within 10 minutes.

Immunization. Several methods of immunization have been attempted. Considerable work has been done in California by Beach who has obtained satisfactory results from a vaccine made by emulsifying material from the lesions on the skin and mucous membranes in physiological salt solution and heating the same at a temperature of 55°C.

POX VIRUS

The term "pox" is used here to designate those acute, febrile, infectious diseases of man, cattle, sheep, goats, horses and hogs, characterized by a vesicular exanthema, and known respectively as "small-pox," "cow-pox," "sheep-pox," "goat-pox," "horse-pox" and "swine-pox."

These diseases, insofar as their etiological factors are concerned, are certainly very closely related. Most authorities are convinced that they are caused by viruses which originated from a single type and have become adapted to the different species of animals in which they cause disease. This close relationship is demonstrated by our well-known method of vaccination against small-pox. Persons vaccinated with cow-pox virus are, as is generally known, protected against small-pox. It is also a fact that a person recovered from small-pox cannot be immediately infected with cow-pox virus.

The pox virus differs from most of the other viruses grouped in this chapter in that it is but slightly filterable. If material containing the virus is passed through a fine filter with the aid of only a minimum amount of pressure or vacuum, the specific virus does not come through. However,

[32] Marx and Sticker, Deutsche medizinische Wochenschrift (Berlin), 1902, 893.

if a coarse filter is employed and considerable pressure used the filtrate will *sometimes* prove infectious.

Prowazek[33] and a number of other investigators maintain that chlamydozoa are concerned in the etiology of the poxes. These minute bodies are 0.2 to 0.5 micron in size, motile and stain with Giemsa's stain. They can be demonstrated in the skin lesions in considerable numbers. If a rabbit is inoculated on the cornea with pox virus there can be demonstrated in the epithelial cells so-called "vaccine-bodies." These are round or crescent-shaped bodies made up of what appears to be a chromatin substance. It is thought that these structures are chlamydozoa surrounded by material resulting from the cell reaction. Others maintain that "vaccine bodies" are merely minute areas of degeneration.

Pox virus is present in the vesicular fluid, in the scabs and, in the beginning of the disease, is present in the blood but only for a very brief period. The excreta of affected animals are only infectious when contaminated with infectious material from the lesions.

Resistance. Pox virus, when protected from air and light and maintained at a low temperature, will retain its virulence for months. It soon becomes avirulent when kept at even moderate temperatures. A temperature of 58°C. destroys it in five minutes. Glycerin is not detrimental to pox virus. This fact is taken advantage of in making vaccine. Glycerin can be added to virus lymph and will destroy bacteria but will not appreciably affect the pox virus. The various disinfectants are effective in the usual dilutions.

Immunization. Jenner's observation that infection with cow-pox virus immunized man against small-pox lead to the widely-employed method of vaccination now known to every school child.

Vaccination of the lower animals against pox is possible but is rarely employed.

[33] Prowazek, Arbeiten aus dem Kaiserlichen Gesundheitsamte (Berlin), 1905, xxiii, 535.

PART VIII

SEROLOGY

CHAPTER XXXIX

Serological Tests Commonly Employed in Veterinary Medicine

The serological tests of interest to the veterinarian include various complement-fixation, agglutination, agglutinin-absorption, precipitation, and conglutination tests. Of these the complement-fixation and agglutination tests are most commonly employed. The agglutinin-absorption tests are of value chiefly in connection with the identification of closely related bacteria. The use of precipitation tests is limited to a very few diseases. While they are of diagnostic value when suitable precipitating sera and properly prepared antigens are employed, such tests have not been used on an extensive scale. The same is true of the conglutination test, it undoubtedly being less frequently employed than any of the others.

The complement-fixation test for glanders is probably the most widely employed and best known test of this type utilized in veterinary medicine. Aside from its use in connection with the diagnosis of glanders, the complement-fixation test has been a highly valuable factor in the diagnosis of bovine and equine infectious abortion, trypanosomiasis, malta fever, etc. The eradication of dourine in the United States has been made possible largely through the practical application of the complement-fixation phenomenon. Further, such test has proved a valuable asset in connection with the examination of animals imported into the United States from countries where trypanosomiases occur. Several instances are on record where apparently normal animals presented for entry into the United States were identified as carriers of trypanosomes, through the use of the complement-fixation test. In the Philippine Islands the author has found this test highly valuable for the identification of carriers of the surra trypanosome.

In addition to its use in the diagnosis of disease, the complement-fixation phenomenon can be often utilized for the identification of various organisms and their by-products.

Agglutination tests can be employed in connection with a number of diseases of the lower animals. The technic of such tests is relatively simple, being far less complicated than that of the complement-fixation test. In diseases where both tests can be utilized the agglutination method is commonly employed because of its simplicity. However, there are some instances in which an animal affected with a certain disease will

react to one test and not to the other. For example, in chronic glanders the agglutination test is frequently negative whereas with the complement-fixation technic positive results are obtained. On the other hand, in very early glanders the agglutinins are present in the blood stream in detectable numbers several days before the complement-fixing bodies appear, hence, if serum from an animal in such early stage is subjected to the complement-fixation test the reaction may be negative whereas an agglutination test might yield positive results. For this reason, in work with glanders, serum specimens are frequently subjected to both tests. Where both the complement-fixation and agglutination tests can be utilized for the diagnosis of a particular disease, and where there is no appreciable difference in reliability, the agglutination method is ordinarily employed.

Agglutination tests of particular interest in veterinary medicine include those for glanders, bovine and equine infectious abortion, Salmonella affections and malta fever.

Precipitation tests can be employed for the diagnosis of glanders, anthrax and swine erysipelas.

In veterinary medicine the conglutination test has been utilized to a limited extent in connection with the diagnosis of glanders.

THE COMPLEMENT-FIXATION TEST

Before taking up the technic of the complement-fixation test it will probably be well to review a little of the theory of the phenomenon.

In discussing the haptines of Ehrlich's Third Order it was indicated that, when properly stimulated, the body is capable of producing several types of cytolytic antibodies. The type which is produced as a result of body reaction against bacteria, and which is capable, in the presence of complement, of causing bacteriolysis or breaking down of the bacterial cell, is termed *bacteriolytic amboceptor*. Where red blood corpuscles have served as the stimulus, the type of antibody produced is designated *hemolytic amboceptor* and is capable, in the presence of complement, of liberating the hemoglobin of blood cells of the particular type which gave rise to the amboceptor.

Putting this part of the theory to a practical test we find that if a little serum from a horse affected, for example, with glanders, is placed in a test tube with a suspension of Pfeifferella mallei (antigen) and a little fresh, normal guinea pig serum added to supply complement, after a short period of incubation at body temperature bacteriolysis will have occurred. The serum of the glandered horse, as a result of the infection, contains bacteriolytic amboceptors specific for Pfeifferella mallei and these, in the presence

of complement, produce bacteriolysis. In the process the complement is linked up with the amboceptor and thus bound or "fixed." This combination of serum, bacterial suspension or extract of bacteria (antigen) and complement constitutes what is known as the *bacteriolytic system* of the complement-fixation test.

The purpose of the complement-fixation test is to demonstrate the presence or absence of specific bacteriolytic amboceptors in the serum under test. If reactions occurring with the combination of serum, antigen and complement were discernible to the naked eye there would be nothing more to the test. Such reactions, however, are not thus discernible. It is, therefore, necessary to employ some means of indicating what has happened. For this purpose we make use of what is termed the *hemolytic system.* The following paragraph explains same.

If a rabbit is injected with washed blood cells from an animal of another species, say a sheep, hemolytic amboceptors are produced. A little serum from such rabbit placed in a test tube containing a suspension of washed, sheep, red blood cells and fresh guinea pig serum for complement, after a short period of incubation will result in hemolysis of the blood cells. This combination of hemolytic amboceptor, complement and blood cells constitutes the *hemolytic system* of the complement-fixation test. The reaction is analogous to that occurring in the bacteriolytic system but, further, the occurrence of hemolysis is readily discernible to the naked eye.

It will be noted that complement, a very unstable, thermolabile agent, normally present in the serum of all animals, is a necessary factor common to both the bacteriolytic and hemolytic systems. This fact makes it possible to utilize the hemolytic system as an indicator of what happens in the bacteriolytic system.

If a definite amount of complement is placed in a tube with a given amount of bacterial antigen and serum containing bacteriolytic amboceptor, and the mixture incubated for a short period of time, all of the complement is bound or fixed in the reaction which results. Then, if a quantity of hemolytic amboceptor and corresponding red blood cells are added to the tube no hemolysis will occur because there is no free complement available. Such reaction indicates that the serum, used in the first part of the test with the antigen and complement, contained bacteriolytic amboceptors specific for the particular antigen used. If a glanders antigen was employed the test is considered positive for glanders.

Now suppose there are placed in a test tube and incubated for a short time, serum from an animal free of glanders, some glanders antigen and a definite amount of complement. There are no bacteriolytic amboceptors

in the serum specific for the glanders antigen, hence, no reaction takes place and the complement remains free. If hemolytic amboceptor and blood are now added the complement already in the tube is free to activate the hemolytic amboceptor and hemolysis results. Such reaction thus indicates an absence of specific bacteriolytic amboceptor in the serum tested and signifies an absence of glanders.

These various reactions are diagrammatically represented by Figure 82.

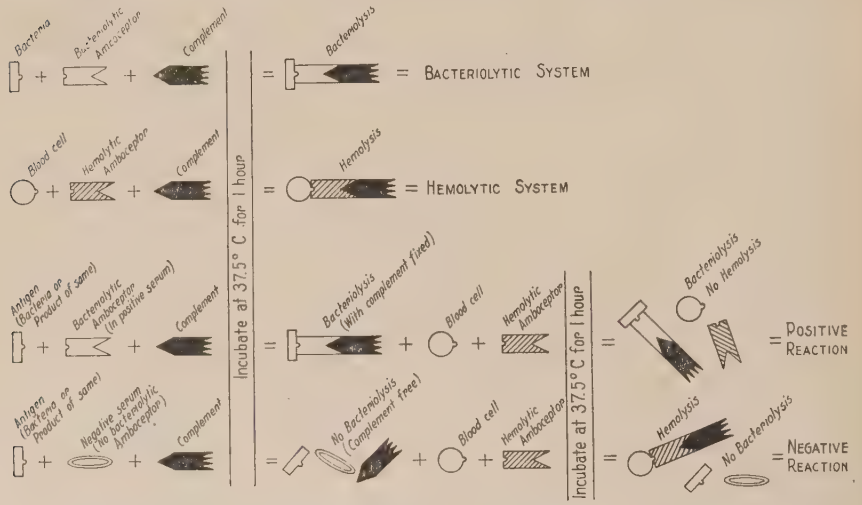


Fig. 82. Diagrammatic Representation of the Reactions Occurring in the Complement-fixation Test

THE COMPLEMENT-FIXATION TEST FOR GLANDERS

As previously stated, in veterinary medicine the complement-fixation test for glanders is, undoubtedly, the most commonly employed of such tests. For this reason, in describing the technic of the complement-fixation test we will use the glanders test as an example. The technic for other complement-fixation tests, in the main, is identical. The difference lies principally in the character of the antigen.

In carrying out the complement-fixation test for glanders, the following equipment and reagents are necessary:

Test tubes, 150 x 15 mm.
Test tube racks, 12 holes front and back
Pipettes, 10 cc. graduated in 0.10 cc.
Pipettes, 1 cc. graduated in 0.01 cc.

Graduate, 100 cc.
Tubes, centrifuge, glass, usually 50 cc.
Flasks, Erlenmeyer, several sizes
Water bath or hot air incubator maintained at 37.5°C.
Water bath, inactivating, maintained at 57.5°C. for horse serum and 62°C. for mule serum
Centrifuge
Physiological salt solution
Antigen, glanders
Complement, fresh, normal guinea pig serum
Hemolytic amboceptor, anti-sheep-cell
Sheep blood cells
Positive glanders serum
Negative serum (normal horse serum)
Serum to be tested

The glassware used in the test need not be sterile but should be scrupulously clean. Of the reagents, the antigen, hemolytic amboceptor and physiological salt solution are prepared and kept on hand as stock reagents. Known positive and negative sera, likewise, can be kept on hand for a considerable period of time. The complement and sheep blood cells must be obtained fresh. The blood cells will keep several days in the ice-box but the complement is obtained fresh for each run of tests.

Dilutions of amboceptor, complement and antigen and the suspension of blood cells are all made with sterile physiological salt solution (0.85 per cent sodium chloride in distilled water).

Hemolytic Amboceptor. Anti-sheep-cell amboceptor is prepared by injecting one or more rabbits with several doses of washed blood cells from a sheep. It is always good policy to start with several rabbits because an animal is occasionally lost as a result of the process and, further, every rabbit treated does not produce amboceptor of desirable titre.

The sheep blood is obtained by bleeding 75 to 100 cc. of blood from a normal sheep into a flask containing 25 to 35 cc. of 0.20 per cent sodium citrate in physiological salt solution, or into a flask containing beads for defibrination of the blood. The author prefers to defibrinate the blood. The blood is simply collected in a flask containing a few glass beads and defibrination accomplished by shaking for about ten minutes.

The next step is the washing of the cells. If the cells have been collected in citrate solution the mixture is poured into 50 cc. glass centrifuge tubes and centrifugalized at two to three thousand revolutions per minute for 15 minutes. Defibrinated blood is first filtered through a layer or two of sterile gauze to remove the fibrin and is then centrifugalized. The supernatent fluid is pipetted off by means of a pipette equipped with a rubber

bulb or, if a vacuum line is available, a wash bottle connected to same can be equipped with a piece of rubber tubing and pipette and such apparatus used to draw off the wash water from the cells. After drawing off the first fluid the tube or tubes containing the "packed" cells are filled with sterile physiological salt solution and the mixture poured back and forth several times between the tube and a sterile flask to insure thorough washing. The mixture is then centrifugalized again for 15 minutes. The washing process is repeated in this manner until the cells have been subjected to 5 or 6 washings. After drawing off the last wash fluid, one part of packed cells is diluted with an equal part of sterile physiological saline solution and this suspension used to inject the amboceptor rabbits. If the rabbits are to be injected intravenously 2 parts of salt solution are added to 1 part of cells.

The rabbits may be injected either intraperitoneally or intravenously. A larger percentage of rabbits will produce high titre amboceptor when injected intravenously than when given intraperitoneal injections. Losses, however, are much higher with the intravenous method. The author usually employs the intraperitoneal method and has experienced no difficulty in producing very satisfactory amboceptor.

When the intravenous route is employed four injections of a $33\frac{1}{3}$ per cent suspension of packed cells are administered in amounts of 1.00, 1.50, 2.00 and 3.00 cubic centimeters, respectively, at 5-day intervals. When given intraperitoneally four injections of 1.00, 2.00, 4.00 and 6.00 cc., respectively, of a 50 per cent suspension of cells in physiological salt solution are given at 5-day intervals. One week after the last injection a small amount of blood is obtained from each rabbit by bleeding from the ear. The serum is separated and the hemolytic value of same established by titration tests. Rabbits whose blood sera show satisfactory titre (1:1000 or more) are bled to death from the heart under ether anesthesia. If the blood serum of one or more of the treated rabbits proves to be of low titre on the preliminary test, additional injections of blood cells may be given. Further injections, however, do not always materially improve the titre of the rabbit's blood. In such cases the rabbits are rejected.

After the blood is collected from the high-titre rabbits the clear serum is removed from the clots, pooled and preservative added. Either phenol or glycerin can be utilized for the preservation of the product. If phenol is employed it is added in the proportion of 1 part of a 5 per cent solution to 9 parts of the serum. When glycerin is used one part of same is added to an equal part of serum. Of the two preservatives the glycerin is preferable. After the preservative has been added to the pooled serum it is

titrated to determine its hemolytic value. Following this it is put up in suitable ampoules or homeopathic phials and properly labeled to indicate titre, etc. When stored in the refrigerator such amboceptor will keep several years with only a gradual loss in titre.

Titration of the Hemolytic Amboceptor. To titrate the hemolytic amboceptor set up a test tube rack with 12 tubes. There must then be at hand fresh complement (guinea pig serum) titrated as hereinafter described and diluted so that 1 cc. contains $1\frac{1}{2}$ units; a 3 per cent suspension of washed blood cells from a sheep; a 1:1000 dilution of the amboceptor to be titrated, and physiological saline solution. The dilutions of complement and ambo-

TABLE 5

Amboceptor titration

TUBE NUMBER	AMBOCEPTOR (1:1000)	COMPLEMENT	BLOOD CELLS	SALT SOLUTION
	cc.	*cc.*	*cc.*	*cc.*
1	1.00	1.00	1.00	2.00
2	0.90	1.00	1.00	2.00
3	0.80	1.00	1.00	2.00
4	0.70	1.00	1.00	2.00
5	0.60	1.00	1.00	2.00
6	0.50	1.00	1.00	2.00
7	0.40	1.00	1.00	2.00
8	0.30	1.00	1.00	2.00
9	0.20	1.00	1.00	2.00
10	0.10	1.00	1.00	2.00
11	1.00	0	1.00	2.00
12	0	1.00	1.00	2.00

ceptor and suspensions of blood cells are all made with physiological salt solution. Place these various reagents in the test tubes in accordance with the outline in Table 5. The tubes are then shaken and placed in the water bath or incubator for one hour. It will be noted that the eleventh tube serves as a control on the amboceptor. It should indicate that the amboceptor is incapable of causing hemolysis in the absence of complement. The twelfth tube contains the complement control and should show that the complement is not in itself hemolytic.

After the expiration of the hour period of incubation the tubes are examined and the smallest amount of the hemolytic amboceptor which has caused complete hemolysis of the blood cells is determined. This amount is known as the *unit of amboceptor*. In recording the unit of amboceptor the figures obtained from the titration test are reduced so as to indicate the

dilution of the concentrated amboceptor which must be made to have 1 unit in 1 cc. of the dilution. If, for example, it is found that 0.50 cc. of the 1:1000 dilution of amboceptor is the smallest amount causing complete hemolysis of the blood cells then 1 cc. of a 1:2000 dilution of the amboceptor would contain 1 unit; if it was found that 0.80 cc. of the 1:1000 dilution was the smallest amount causing complete hemolysis then 1 cc. of a 1:1250 dilution would contain one unit. In carrying out the complement-fixation test 2 units of amboceptor are employed, the dilution of the stock amboceptor being made so that this amount is contained in 1 cc. Thus, if the stock of amboceptor is labelled to indicate that 1 cc. of a 1:2000 dilution contains 1 unit we would make a 1:1000 dilution so that 1 cc. would equal 2 units.

Complement. Fresh serum from normal guinea pigs is universally employed for complement in the various complement-fixation tests. In obtaining complement, serum from at least 2 guinea pigs should be obtained and pooled. There is quite a little variation in the complement content of the sera of different guinea pigs so in order to obtain the best results pooled sera from several pigs should be utilized.

The guinea pig blood may be collected by one of several methods. Where large numbers of tests are run routinely and the supply of guinea pigs is plentiful, it is sometimes more economical to bleed pigs to death rather than to maintain a large number of bleeders which are bled successively at intervals of 4 or 5 weeks or more. If the guinea pigs are to be bled to death for complement the common procedure is to shave the hair from the throat region, stun the animal by a smart blow on the head and then sever the carotids and jugulars with a sharp knife, catching the blood in test tubes. A small, short-stem funnel in the test tube will facilitate catching the blood.

If the complement is to be obtained from pigs which are not to be killed proceed as follows: Shave the hair over the left chest and then anesthetize the animal with ether. Paint the shaved area over the chest with iodine and when the animal is completely under the anesthetic introduce a needle attached to a 10 cc. glass barrel syringe into the heart and withdraw 8 or 10 cc. of blood. The point at which the needle is introduced is best determined by catching the chest between the thumb and forefinger and determining where the heart beat can be felt most distinctly. When such point is ascertained place the needle in the intercostal space just over such area and carefully force it through the chest wall and into the heart. After a little practice no difficulty is experienced in hitting the heart and when skillfully done the losses from the operation are not large.

After drawing the blood from the heart it is discharged from the syringe into a test tube. If the serum is to be used immediately the blood is allowed to coagulate and the clot is then loosened from the walls of the test tube with a clean glass rod or piece of stiff wire. It is then good practice to place the tube or tubes containing the blood in the incubator at 37.5°C. for 15 or 20 minutes to facilitate contraction of the clot. After such period the tubes are removed, placed in the centrifuge and the same operated at 1500 to 2000 revolutions for 20 minutes. On removing the tubes from the centrifuge a goodly yield of clear serum will be found separated from the clot. This is either poured or pipetted off into a clean tube taking care not to carry over blood cells. If even a few cells are carried over with the serum centrifugalization should be again resorted to in order to provide a serum absolutely free of cells. This is very important because a few guinea pig blood cells in the serum will greatly reduce its complement value through inhibitory action.

As indicated, guinea pig serum intended for complement must be obtained fresh. By this is meant that it should be obtained the same day it is used or not sooner than the day before. Satisfactory results are obtained with complement if it is bled the afternoon preceding the day of the test and kept at refrigerator temperature until used. When bled the day before the serum should be left on the clot until ready to use it.

Titration of the Complement. For the titration of complement place 16 test tubes in a test tube rack. Then have ready a 3 per cent suspension of washed blood cells from a sheep; a quantity of hemolytic amboceptor so diluted that 1 cc. contains 2 units, and physiological salt solution. Make a 4 per cent dilution of the guinea pig serum by adding 0.50 cc. of same to 12 cc. of physiological salt solution. Place these reagents in the test tubes in accordance with the outline in Table 6. After the ingredients have all been placed in the tubes they are shaken and incubated at 37.5°C. for one hour. At the expiration of this period the tubes are examined and the smallest amount of the complement dilution causing complete hemolysis of the blood cells is determined. This amount is known as the *unit.* In conducting the complement-fixation test $1\frac{1}{2}$ units of complement are employed and the dilution is made so that this amount will be contained in 1 cc. Such figure is arrived at by merely multiplying the figure representing the unit of complement by 4 and then adding 50 per cent of such amount. For example, we find that 0.70 cc. of the 4 per cent dilution of complement is the unit or smallest amount of the dilution that resulted in complete hemolysis of the blood cells. Thus $0.70 \times 4 = 2.8$. To have 1 unit of complement in 1 cc. of fluid we would, therefore, use a 2.8 per cent

dilution of the guinea-pig serum. However, we use $1\frac{1}{2}$ units of complement in the test so in order to have that amount in 1 cc. of fluid we would make a 4.2 per cent solution of the straight complement.

Antigen. Antigen for the complement-fixation test for glanders is prepared in the following manner: Inoculate a number of 3 per cent glycerin agar slants, of pH 6.8 reaction, with Pfeifferella mallei and incubate them for 48 hours. While one strain of the glanders organism will suffice, it is better policy to utilize several strains in the preparation of antigen. After growing for 48 hours carefully examine to determine purity of growth. Now add to each tube 5 or 6 cc. of sterile *distilled water* and with the aid

TABLE 6
Complement titration

TUBE NUMBER	COMPLEMENT	AMBOCEPTER	BLOOD CELLS	SALT SOLUTION
	cc.	*cc.*	*cc.*	*cc.*
1	1.00	1	1	2.00
2	0.95	1	1	2.00
3	0.90	1	1	2.00
4	0.85	1	1	2.00
5	0.80	1	1	2.00
6	0.75	1	1	2.00
7	0.70	1	1	2.00
8	0.65	1	1	2.00
9	0.60	1	1	2.00
10	0.55	1	1	2.00
11	0.50	1	1	2.00
12	0.45	1	1	2.00
13	0.40	1	1	2.00
14	0.35	1	1	2.00
15	0	1	1	2.00
16	1.00	0	1	2.00

of a platinum loop wash the growth from the agar slants. The next step is to transfer the washings from the various slants to an Erlenmeyer flask of such size that the pooled bacterial suspension will not fill the flask more than one-third full. A good way to accomplish the transfer of the suspension is to place a long-stem funnel in the Erlenmeyer flask and then carefully pour the culture washings into the funnel. The discarded culture tubes should be carefully placed in a suitable container for immediate sterilization. After the last culture washing is poured over the funnel is carefully removed, without contaminating the upper portion of the flask, and placed in the container with the discarded tubes for sterilization. The neck and

upper portion of the flask are then well flamed and the cotton stopper inserted. With as little shaking as possible, the flask is transferred to a water-bath maintained at 65°C. and submerged so that the water comes up near the top of the flask's neck. A clamp attached to an iron stand can be adjusted to hold the flask in proper place in the bath. The bacterial suspension is subjected to the temperature of the water bath (65°C.) for one hour. At the expiration of such period it is carefully removed and the neck of the flask again well flamed.

This killed suspension is then diluted with sterile distilled water containing sufficient phenol to make the dilution of antigen contain 0.50 per cent phenol. There is no set rule governing the amount of water to be added at this point. Care should be taken not to add too much. A good heavy suspension should be the result. If the product is diluted too much a weak antigen will be the result. On the other hand, the distilled water causes a disintegration of Pfeifferella mallei and if the suspension is exceedingly heavy the process of disintegration is less complete.

The next step is to place the antigen in a shaking machine and shake it for 1 or 2 hours on 5 or 6 consecutive days. At the end of this time the preparation is placed in heavy glass centrifuge tubes and centrifugalized at high speed (4 to 5 thousand revolutions per minute) for one hour. At the end of this process the slightly cloudy, supernatent fluid is drawn off and constitutes the stock antigen.

Antigen Titration. In carrying out the antigen titration a test tube rack containing 24 test tubes (12 front and back) is employed. The following reagents are required: Titrated complement, $1\frac{1}{2}$ units being contained in each cubic centimeter of fluid; hemolytic amboceptor diluted so that 2 units are contained in 1 cc. of fluid; 3 per cent suspension of washed blood cells from a sheep; physiological saline solution; known positive glanders serum; known negative serum, and a dilution of the antigen to be titrated. The extent to which a new stock antigen should be diluted is problematical until after the first attempt at titration. The proper dilution will vary from 1:10 to 1:50 or more. If one does not happen to strike the proper dilution at the first titration the process must be repeated until same is determined.

The titration is carried out as follows: Pipette 2 cc. of sterile physiological salt solution into each of the 24 test tubes. Starting at the left of the front row of tubes put 0.15 cc. of the known positive glanders serum in each of 10 consecutive tubes omitting it in the last 2 tubes in the front row. Go to the back row and repeat the procedure but use the known negative serum in place of the positive. Mark the 10 positive and 10

negative tubes for identification and place them in a water bath at 57.5°C. for 20 minutes. This is done to *inactivate* the sera, i.e., to destroy any complement it may contain. This heating process not only destroys any com-

TABLE 7

Antigen titration

Front row of tubes

TUBE NUMBER	SALT SOLUTION	POSITIVE SERUM	ANTIGEN	COMPLEMENT		HEMOLYTIC AMBOCEPTOR	BLOOD CELLS	
	cc.	cc.	cc.	cc.		cc.	cc.	
1	2	0.15	0.05	1	Incubate	1	1	Incubate
2	2	0.15	0.10	1	1 hour	1	1	1 hour
3	2	0.15	0.20	1	at	1	1	at
4	2	0.15	0.30	1	37.5°C.	1	1	37.5°C.
5	2	0.15	0.40	1		1	1	
6	2	0.15	0.50	1		1	1	
7	2	0.15	0.60	1		1	1	
8	2	0.15	0.70	1		1	1	
9	2	0.15	0.80	1		1	1	
10	2	0.15	0	1		1	1	
11	2	0	0	1		1	1	
12	2	0	0	0		1	1	

Back row of tubes

TUBE NUMBER	SALT SOLUTION	NEGATIVE SERUM	ANTIGEN	COMPLEMENT		HEMOLYTIC AMBOCEPTOR	BLOOD CELLS	
	cc.	cc.	cc.	cc.		cc.	cc.	
1	2	0.15	0.10	1	Incubate	1	1	Incubate
2	2	0.15	0.20	1	1 hour	1	1	1 hour
3	2	0.15	0.40	1	at	1	1	at
4	2	0.15	0.60	1	37.5°C.	1	1	37.5°C.
5	2	0.15	0.80	1		1	1	
6	2	0 15	1.00	1		1	1	
7	2	0.15	1.20	1		1	1	
8	2	0.15	1.40	1		1	1	
9	2	0.15	1.60	1		1	1	
10	2	0 15	0	1		1	1	
11	2	0	0.80	1		1	1	
12	2	0	0	1		0	1	

plement present but also destroys certain inhibitory substances present in a number of sera. The whole serum can be inactivated before it is distributed in the tubes but there are advantages in inactivating it in the salt solution.

The tubes are returned from the inactivating bath to their proper places in the rack and the titration continued as indicated in Table 7. It will be noted that varying amounts of antigen are added to the first 9 tubes in the front row containing positive serum. The tenth tube in this row serves as a control on the positive serum employed, only such serum and the ingredients of the hemolytic system being present in this tube. If the positive serum happens to be one which will, in itself, inhibit hemolysis this tube will reveal such fact. The eleventh tube in the front row is also a control tube and contains only the hemolytic system. This serves to show that the hemolytic system is or is not working properly. The last tube in this row is likewise a control tube and serves to indicate that the hemolytic amboceptor is not, in itself, capable of causing hemolysis. In the back row it will be observed that the first 9 tubes containing negative serum have each received an amount of antigen equal to twice that placed in the corresponding front tubes containing positive serum. The tenth tube in the rear row contains the negative serum and hemolytic system and serves to indicate whether or not such serum is capable of inhibiting hemolysis. The eleventh tube in this row serves as the antigen control. It contains the hemolytic system and an amount of antigen equal to the highest amount (0.80 cc.) used with the positive serum. This control is to indicate whether or not the antigen is, in itself, capable of inhibiting hemolysis. The last tube in the back row contains only the complement and suspension of blood cells and serves to indicate whether or not the complement, is in itself, hemolytic.

It will be noted that two incubation periods are employed in the antigen titration. The first follows the addition of serum, complement and antigen, and is for the purpose of facilitating the union of antigen, bacteriolytic amboceptor and complement (the latter being fixed) in the tubes containing the positive serum. The second incubation period facilitates the reaction of the hemolytic system.

After the second incubation period the tubes are examined and the smallest amount of antigen resulting in complete fixation of complement is determined. This is represented by the tube in the front row of the rack (with positive serum) containing the smallest amount of antigen and showing no trace of hemolysis. For example, the first tube in the front row containing 0.05 cc. of antigen shows about 25 per cent hemolysis, the second tube containing 0.10 cc. of antigen shows no evidence of hemolysis whatever and in such event it is obvious that there would be no hemolysis of the blood cells in the tubes which received greater amounts of antigen. Thus the *unit* of antigen, in this case, is 0.10 cc. of the particular dilution

employed, *provided* that a similar amount of the antigen (0.10 cc.) does not interfere with hemolysis in the presence of negative serum. This latter is determined by examining the tube in the back row containing 0.10 cc. of antigen and negative serum.

We speak of the *range* of an antigen. This is represented by the difference between the smallest amount of antigen which results in complete fixation of complement in the presence of positive serum and the largest amount which can be employed without inhibiting hemolysis in the presence of negative serum. Some types of antigens have relatively short ranges while others have long ranges. Glanders antigen, prepared as described, usually has a long range.

In the complement-fixation test proper 3 units of antigen are employed provided twice that amount (6 units) does not interfere with hemolysis in the presence of negative serum. In diluting stock antigen for the complement-fixation test, the amount of physiological salt solution added should be such that the 3 units are contained in 0.50 cc. of the dilution.

The Actual Test. If a large number of blood specimens are to be tested two tubes are employed for each specimen, one for the test proper and the other for the serum control. If only a few specimens are tested two, or even three, dilutions can be run. This is not absolutely necessary but running 2 or 3 tubes per specimen, in addition to the serum control tube, frequently gives more information than a single tube and, further, such procedure is an additional check against errors in technic. Where a total of only 2 tubes is employed for each specimen a test tube rack holding 24 test tubes (12 front and back) is set up and 2 cc. of physiological salt solution added to each tube. The serum specimens are added in amounts of 0.15 cc. putting that amount of a specimen in both a front and corresponding back tube. The tubes should be properly numbered for identification. If two tubes are used in addition to the control tube 0.15 and 0.10 cc., respectively, of serum are used for the test proper and 0.15 cc. for the serum control. If 3 tubes are employed in addition to the control tube 0.15, 0.10 and 0.05 cc. of serum, respectively, are tested, the control tube receiving 0.15 cc.

After the serum specimens have been added in proper amounts in the different tubes, properly marked for identification, they must be inactivated. If the serum is from a horse the specimens are placed in the water-bath maintained at 57.5°C. and allowed to remain in same for 20 minutes. In the case of mule serum exposure to a temperature of 62°C. for 40 minutes is required. This is necessary because most mule serum normally contains an anti-complementary principle which results in the inhibition of hemoly-

sis. This principle is not destroyed by the temperature employed for the inactivation of horse serum. The temperature (62°C.) at which the mule serum must be heated is dangerously near that which destroys specific complement-fixing bodies, hence, great care should be exercised to insure against exceeding same. After the inactivation remove the tubes from the inactivating bath and proceed to add the following reagents in the order and amounts indicated:

1. *Antigen*—3 units diluted so that same is contained in 0.50 cc. of physiological saline solution. This is only added to each of the tubes in the front row, *not* to the serum controls in the back row.
2. *Complement*—1½ units of complement diluted with physiological saline solution so that amount for test is 1 cc. This is added to all tubes.
3. *Incubate* 1 hour in water bath or hot air incubator at 37.5°C.
4. *Hemolytic amboceptor.* After the expiration of incubation period add 2 units of anti-sheep-cell hemolytic amboceptor so diluted that same is contained in 1 cc. of physiological saline solution. This is added to all tubes.
5. *Blood cells.* Add 1 cc. of a 3 per cent suspension of washed, red blood cells from a sheep. This is added to all tubes.
6. *Incubate* 1 hour in water bath or hot air incubator at 37.5°C.

It is understood, of course, that the complement is titrated just prior to use and in the test proper it is used with the same dilution of amboceptor and same suspension of blood cells as figured in the complement titration. The amboceptor and glanders antigen employed are titrated periodically but the procedure need not be carried out with every run of tests when conducted at frequent intervals.

With each run of tests a known positive glanders serum and a known negative specimen must be included as controls.

At the end of the second incubation period the test is "read." A typically positive reaction is indicated by an entire absence of hemolysis in the tube or tubes containing serum and antigen whereas the corresponding serum control tube which contains no antigen shows complete hemolysis of the blood cells. A typically negative reaction is indicated by complete hemolysis of the blood cells in the tubes containing antigen and in the control tube. Intermediate between the typically positive and negative responses, partial reactions are occasionally obtained. The interpretation of these frequently offers difficulty. In very early cases of glanders complete fixation of complement may not be obtained. Then, if the animal has received injections of mallein (especially for the first time), such inoculation gives rise to the formation of complement-fixing bodies. Serum specimens which are more or less decomposed or which contain appreciable amounts of hemolyzed blood cells are *inhibitory* (inhibit hemolysis). Some

specimens of mule serum, even after inactivation at 62°C., may still be slightly inhibitory.

In recording the results of the test *plus* and *minus* symbols are commonly employed as follows:

++++ = no hemolysis, 100 per cent fixation of complement
+++ = approximately 75 per cent fixation of complement
++ = approximately 50 per cent fixation of complement
+ = approximately 25 per cent fixation of complement
+− = less than a single plus reaction
− = complete hemolysis, no fixation of complement

These reactions are illustrated by Figure 83.

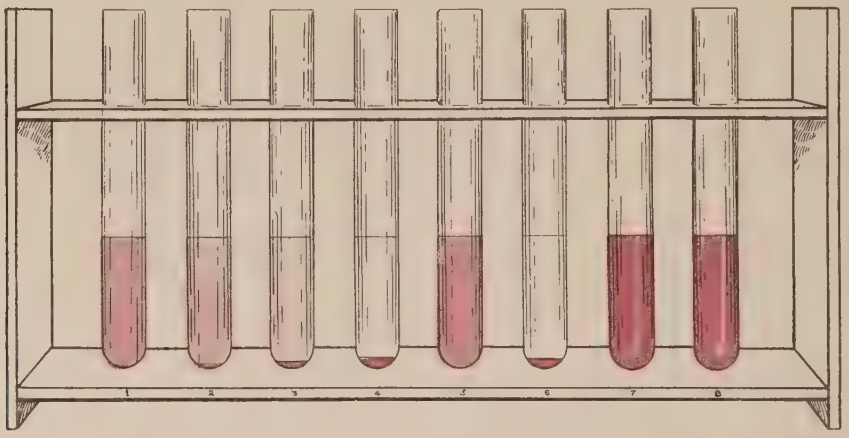


FIG. 83. COMPLEMENT-FIXATION REACTIONS

1, + reaction; *2*, ++ reaction; *3*, +++ reaction; *4*, ++++ reaction; *5*, negative control; *6*, positive control; *7*, antigen control; *8*, hemolytic system.

COMPLEMENT-FIXATION TEST FOR TRYPANOSOMIASIS

The complement-fixation test for trypanosomiasis is a group reaction. That is to say, the serum from an animal infected with any type of trypanosome will give a positive complement-fixation reaction with any trypanosome antigen. Thus, in localities where several types of trypanosome diseases are prevalent this test will not distinguish between them.

The technic of the complement-fixation test for the diagnosis of trypanosome infections is identical with that described for the glanders test with the exception of the preparation of the antigen.

Several types of antigens have been prepared and utilized in connection

with this test. In the United States, Mohler, Eichhorn, Traum and Buck,[1] early in connection with the serological diagnosis of dourine, employed an antigen prepared by making a saline suspension of emulsified spleen tissue from rats dead of dourine. In connection with dourine investigations in Canada, Watson employed an antigen consisting of a suspension of pure trypanosomes. This latter type of antigen soon supplemented the spleen tissue antigen in tests for the diagnosis of dourine in the United States. The method of preparing the antigen commonly employed by the United States Bureau of Animal Industry has been described by Reynolds and Schoening.[2] The author has employed essentially the same method for both dourine and surra tests and has found it entirely satisfactory. The method is undoubtedly applicable for the preparation of antigen for any type of trypanosomiasis.

Preparation of Antigen. Ten or more white rats are inoculated subcutaneously or intramuscularly with blood from a previously infected rat whose blood is found to contain numerous trypanosomes. Rats are employed because trypanosomiasis in such rodents runs a short, acute course, the trypanosomes in the circulation rapidly increasing so that in 4 or 5 days the blood is swarming with them. Death of the rats would ordinarily occur in 5 to 7 days but they are not allowed to die. When the blood is found to be swarming with the organisms the rats are bled to death by severing the carotid arteries and jugular veins after stunning the animals with a blow on the head. The blood is caught in approximately an equal part of 0.20 per cent sodium citrate in physiological saline solution. After the blood has been collected from all the rats it is placed in one or more centrifuge tubes and centrifugalized at high speed for 45 minutes. Examination will show the blood cells packed in the bottom of the tube and on top of same a thin, white, upper stratum consisting largely of trypanosomes. The supernatent fluid is drawn off taking care not to draw up any of the layer of trypanosomes. At one time it was the practice to carefully collect the white layer of trypanosomes with a small pipette and discard the rest of the material. It has been found, however, that a good many trypanosomes are contained in the mass of packed cells so in place of removing the white layer we add distilled water to the cell mass in sufficient quantity to lake the red blood cells. The mixture is centrifugalized at high speed for 45 minutes. It will then be found that the trypanosomes have been "thrown down" in the bottom of the tube together with a little cell debris and possibly a few cells which escaped laking. The supernatant

[1] Mohler, Eichhhorn, Traum and Buck, Jour. Agric. Research, vol. i, p. 99, 1913.
[2] Reynolds and Schoening, Jour. Agric. Research, vol. xiv, no. 13, p. 573, 1918.

fluid is drawn off, taking care not to get too close to the trypanosome mass. A little sterile physiological saline solution is added to the tube for the purpose of washing the trypanosomes and the centrifugalization process repeated. After this wash fluid is drawn off one part of 50 per cent glycerin in physiological salt solution is added to 1 part of packed trypanosomes. This constitutes the stock antigen. If it is placed in a tightly stoppered test tube and maintained at a temperature around the freezing point, or even in a frozen state, it can be kept for a month or more. The author places the tube of antigen in an ice-salt mixture and keeps same in a refrigerator at low temperature, renewing the ice-salt mixture every day.

For use the stock antigen is diluted from 1:10 to 1:20 with sterile physiological saline solution and then titrated. With this type of antigen, titration of same is a necessary preliminary step with each run of tests.

Titration of the Antigen. The method of titrating this antigen does not differ from that employed with glanders antigen. The range obtained with the average trypanosome antigen, however, is not as great as that noted with glanders antigen. For this reason, in the titration some laboratories do not double the amounts of antigen in the negative serum tubes but use the same amounts as employed in the front row of tubes containing positive serum.

COMPLEMENT-FIXATION TEST FOR BOVINE AND EQUINE INFECTIOUS ABORTION

The tests for these two diseases do not differ from the glanders test except as regards the nature of the antigen.

Preparation of Bovine Infectious Abortion Antigen. In bovine infectious abortion the causative factor is Alcaligines abortus. Thus, the antigen for the test of cattle is made from such organism. It can be prepared by growing the organism on solid medium for 48 hours, washing off the growth with sterile physiological salt solution and killing the bacteria by heating the suspension at 60°C. for one-half hour. After the heating process sufficient of a 5 per cent solution of phenol is added to the stock antigen to give a phenol concentration of 0.50 per cent. A titration test is then conducted to determine the proper dilution and titre of the antigen.

Antigen for the complement-fixation test for bovine abortion can also be prepared by growing Alcaligines abortus in bouillon for 48 hours, heating the cultures at 60°C. for one-half hour and then adding sufficient 5 per cent phenol solution to make the concentration of phenol in the antigen 0.50 per cent. For use, such antigen is diluted with 2 to 4 parts of physiological

salt solution and titrated in the same manner as that described for the glanders test.

Preparation of Equine Infectious Abortion Antigen. Equine infectious abortion is caused by Salmonella abortus-equi, the antigen for the complement-fixation test for this disease, obviously, being prepared from cultures of such organism. The best type of antigen is prepared from bouillon cultures. These are grown for 48 hours, heated at 60°C. for one-half hour and then phenolized to the extent of 0.50 per cent, using a 5 per cent solution of phenol. For titration, this antigen is diluted 2 to 4 times with physiological saline solution. The technic of the titration is the same as for glanders.

COMPLEMENT-FIXATION TEST FOR MALTA FEVER

In carrying out the complement-fixation test for Malta fever the general technic described for the glanders test applies. In making the antigen either solid or bouillon cultures of Alcaligines melitensis are used and prepared in a manner identical to that described for Alcaligines abortus antigen. For use, the stock antigen is diluted 2 to 4 times with physiological saline solution and titrated according to the same technic specified for the glanders test.

COMPLEMENT-FIXATION TEST FOR ANTHRAX

If a small part of the spleen or liver of an animal dead of anthrax is worked up in a mortar with physiological saline solution and such suspension titrated as an antigen in the presence of anthrax immune serum, a positive complement-fixation reaction can be obtained. While such reaction can be obtained in cases where tissues are available ordinary bacteriological methods suffice, the complement-fixation test not having come into general use.

The complement-fixation test may also be employed for the identification of anti-anthrax serum. The best antigen for this purpose is an old, bouillon culture of low virulence (Pasteur No. 1 or 2 vaccine strains) which has undergone more or less autolysis. Such culture is heated to 60° to 65°C. for one-half to one hour, diluted with 1 to 4 parts of physiological saline solution and then titrated against the anti-anthrax serum. If the latter is from a horse which has been hyperimmunized against anthrax a positive complement-fixation reaction will result. If such serum is then titrated out against several units of the antigen it will usually be found to give complete fixation of complement in small amounts (in quantities as

low as 0.01 to 0.005 cc.). At one time it was thought that the complement-fixation titre of the serum might indicate something of its potency. Such, however, is not the case, it having been definitely demonstrated that the complement-fixing value and potency of a serum do not necessarily run parallel. It is a fact, however, that an animal must receive rather large amounts of anthrax culture before its serum has an appreciable complement-fixation value. The test thus serves to indicate whether or not the serum is from an animal which has received considerable amounts of culture.

IDENTIFICATION OF CLOSTRIDIUM BOTULINUM AND ITS TOXIN BY THE COMPLEMENT-FIXATION TEST

The author has found that Clostridium botulinum and its toxin can be identified in culture and canned food-stuffs by means of the complement-fixation test. Ordinarily immunologic tests with laboratory animals, supplemented in some cases by bacteriological study, constitute the usual routine procedure followed by most laboratories for the identification of Clostridium botulinum and its toxin. While such method is generally satisfactory, it frequently involves considerable time, particularly in instances where the material under examination is contaminated with other organisms besides Clostridium botulinum. If the examination is being made in connection with a suspected outbreak of botulism, the importance of an early diagnosis is, of course, obvious.

Attempts at diagnosing botulism in affected individuals and animals, through the use of the complement-fixation test, have been generally unsuccessful, due, undoubtedly, to the fact that the disease in most instances runs a short, acute course, which, in serious cases, terminates in death before complement-fixing bodies are present in detectable numbers in the blood stream. However, in the case of cultures, contaminated canned food-stuffs, etc., the organism or its toxin can be identified by using an antitoxic serum from a hyperimmune animal as a positive serum, and a preparation of the culture or canned food-stuff as antigen in a titration test.

Preparation of Antigens from Cultures of Clostridium botulinum. In preparing cultures for identification by the complement-fixation test, several methods have been employed with good success. If the organism to be identified has been isolated in pure cultures, a dextrose-beef-broth culture (1 per cent dextrose with a small quantity of chopped beef in each tube), 48 hours or more old, is simply sterilized in the autoclave under 18 pounds

pressure for 30 minutes, the fluid poured off and diluted with 1 to 3 parts of physiological salt solution, according to the density of the culture, and titrated.

With broth cultures contaminated with other organisms it is best to test filtrates of the culture. Such cultures should be at least five days old. They are carefully filtered through asbestos wool and then through a Berkefeld filter of "N" porosity. This filtrate is then placed in the Arnold sterilizer for one hour to render harmless the toxin, if present. It is then titrated, undiluted, against Clostridium botulinum antitoxic serum. Such cultural filtrates give exceedingly good results in cases where Clostridium botulinum is present in the original culture. The reactions are clear-cut and there is usually a wide range between the complement-fixing unit with the positive serum and the anticomplementary point with the negative serum.

In place of dextrose-beef-broth containing a small amount of sterile chopped beef, the beef heart, peptic digest medium, recommended by Dubovsky and Meyer[3] can be used. These investigators found that the elaboration of toxin by impure cultures of Clostridium botulinum is excellent in such medium.

Antigen from Contaminated Canned Products. When examining canned food-stuffs for Clostridium botulinum or its toxin the antigens are prepared as follows: In the case of canned vegetables the fluid from same is carefully drawn off into sterile Erlenmeyer flasks with the aid of a pipette and rubber bulb. With some products such as spinach, when the amount of free fluid is small, it is occasionally necessary to express the liquor from the solid material with the aid of a spatula, pressing the mass against the wall of the can. All pipettes and other apparatus coming in contact with the infected material should, of course, be promptly sterilized in the autoclave. With meat products there is usually no appreciable amount of fluid present. In such cases several ounces of the infected material are macerated in a mortar with 50 cc. of distilled water. The mixture is then placed in a funnel containing cheese cloth and the fluid squeezed through the cloth into an Erlenmeyer flask by twisting the free border of the cloth around the soaked material and bringing pressure on it with a spatula. The fluid from the suspected product is filtered through paper and the filtrate sterilized in the autoclave at 18 pounds pressure for 30 minutes. Usually after sterilizing more precipitate is formed. This should be allowed to settle and the supernatant fluid drawn off for the titration test.

[3] Dubovsky and Meyer, Jour. Infect. Diseases, vol. 31, no. 6, December, 1922.

Titration of the Antigens. Clostridium botulinum antigens prepared as described above, are titrated in accordance with the general technic described for the titration of glanders antigen. For positive serum botulism antitoxin is employed while for the negative serum that from a normal horse suffices. The amounts of antigen used in the titration are graduated from 0.05 to 0.80 cc., the same as with glanders antigen. The amounts, however, are not doubled with the negative serum. While a good range is usually obtained with cultures and culture filtrates, the range with antigens prepared from canned food-stuffs frequently is relatively short.

OTHER USES FOR THE COMPLEMENT-FIXATION TEST

The complement-fixation test can frequently be utilized for the indentification of various cultures, bacterins, vaccines and antisera, other than those already referred to.

In the case of a culture, bacterin or vaccine, it is necessary, of course, to have on hand specific serum for the particular organism which one suspects is being dealt with in the culture, bacterin or vaccine under consideration. In such cases an antigen is prepared from the culture, or, in the case of a bacterin or vaccine the straight product may be used as an antigen. Such antigen is then titrated against the serum specific for the organism suspected.

It is obvious, of course, that all types of cultures, bacterins and vaccines cannot be identified by means of the complement-fixation test because it is not possible to prepare suitable sera or to obtain complement-fixation reactions with all organisms. However, the test can be utilized to identify such organisms as Pfeifferella mallei, Alcaligines abortus and melitensis, organisms of the Salmonella and Pasteurella groups, etc., both in culture and in biological products. In the case of closely related species of a particular genus the test will usually not differentiate them.

In many instances the agglutination test can be employed for identification purposes and when such is the case it is usually preferable to the complement-fixation test because of its relative simplicity.

When, the complement-fixation test is used to identify immune serum, an antigen is made from a culture of the organism suspected of having figured in the preparation of the particular serum and titrated against such serum.

THE AGGLUTINATION TESTS

There are two types of agglutination tests commonly employed. One is the microscopic test and is carried out on a microscopic slide and reactions

observed with the microscope; the other is the macroscopic agglutination test and is set up in small (Wassermann) test tubes. As the name indicates the results with the latter test are discernible to the unaided eye.

Microscopic Agglutination Tests. In subjecting a specimen of serum to a microscopic agglutination test prepare a 1:20 dilution of the specimen in physiological saline solution. Place a loopful of the diluted serum on a clean cover-slip and add a loopful of either a 24-hour bouillon culture or a light suspension of an agar culture of the particular organism the serum is to be tested against. The mixture on the cover-slip is then inverted over the depression in a hollow-ground slide properly prepared with vaseline to pre-

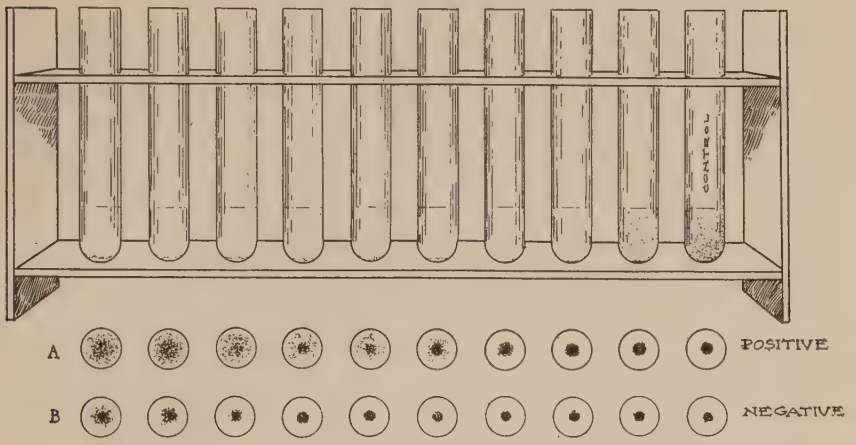


FIG. 84. MACROSCOPIC AGGLUTINATION TEST

a, appearance of material in bottom of tubes in positive case; *b*, appearance of material in bottom of tubes in negative case.

vent evaporation. Place the preparation on the microscope stage and observe with the high power, dry objective for 15 minutes unless agglutination becomes apparent in less time. The test should be controlled by placing a loopful of the bacterial suspension or culture used in a 1:20 dilution of normal serum and observing for evidence of agglutination.

If the agglutination test is employed for the identification of a culture then, of course, a serum known to agglutinate organisms of the species suspected is employed.

The Macroscopic Agglutination Test. This is the type of agglutination test most commonly employed. It not only demonstrates the ability or inability of a serum to agglutinate a certain organism but also indicates

the dilutions in which it will or will not agglutinate. The test merely consists of making a series of dilutions of the particular serum in a group of test tubes and adding to each a definite amount of agglutinating antigen prepared from cultures of the organism the serum is to be tested against. After the antigen is added the mixtures are incubated at 37.5°C. for several hours and placed in the ice-box until the next morning when the results are recorded.

When early readings are desired fairly satisfactory results can be obtained by incubating the mixtures for one or two hours and then placing the tubes in the centrifuge and operating same at rather low speed. The test can then be "read" immediately.

Whether the tubes have been allowed to stand overnight or have been centrifugalized, complete agglutination is indicated by the organisms clumping together and settling out over a considerable portion of the bottom of the tube in the form of a loose, irregular film, leaving the supernatent fluid clear. The absence of agglutination is indicated by only a partial clearing of the fluid and what organisms do settle out are found in the central portion of the bottom of the tube in a compact, regular bordered, circular mass. Intermediate between complete agglutination and an entire absence of same, we recognize various degrees of partial agglutination. Such reactions are indicated by partial clearing of the supernatant fluid and the settling out of a variable number of bacterial clumps in the bottom of the tubes.

Figure 84 illustrates the various agglutination reactions.

The dilutions of serum employed in the macroscopic agglutination tests vary between wide limits with the tests for different diseases. The proper dilutions for several of the most common tests are indicated below.

AGGLUTINATION TEST FOR GLANDERS

As previously indicated the agglutination test for glanders is most valuable in testing sera from horses in the early stages of the disease. The agglutinins make their appearance before complement-fixing bodies but they do not persist like the latter when the disease becomes chronic.

Preparation of Antigen. The first step in the preparation of an agglutinating antigen for glanders is to obtain a suitable culture. Only a small percentage of cultures of Pfeifferella mallei are suitable for agglutination purposes. Having procured a suitable strain, cultures of same are grown on glycerin agar, of pH 6.8 reaction, for 24 to 48 hours. The growth is washed from the agar with sterile, physiological salt solution and heated at 65°C. for one hour to kill the organisms. This suspension should then be diluted

to desired density with more physiological salt solution. The extent of the dilution should, if possible, be determined by comparing the newly made product with a quantity of previously made antigen which has proved satisfactory. If such is not available it is well to make up small amounts of several dilutions and test them with a known positive serum. After determining which of such dilutions is most satisfactory the entire lot of antigen can be properly diluted. After the final dilution formalin is added to the extent of 0.25 per cent. Such antigen, kept tightly stoppered and maintained at refrigerator temperature, will prove satisfactory for a number of months.

Serum Dilutions. In the glanders test 5 dilutions of serum can be employed. The test is set up as follows: Place 6 small (Wassermann) tubes in a rack containing holes through which the bottoms of the tubes can be observed, and in each tube place 1 cc. of sterile, physiological salt solution. Make a 1:50 dilution of the suspected serum and of this add 1 cc. to the first tube in the rack. Mix this thoroughly with the saline solution by drawing it up in the pipette and blowing it back several times. Now carry 1 cc. of this mixture into the second tube and mix same. From the second tube take 1 cc. of the solution and place it in the third tube. Mix same and then withdraw 1 cc. and discard it. In a separate large test tube make a second basic dilution of 1:250 of the original serum by placing 1 cc. of the 1:50 dilution in the tube and adding 4 cc. of physiological saline solution to same. Now go back to where we left the test and in the fourth tube place 1 cc. of the 1:250 dilution just made. Mix this well and carry 1 cc. of such dilution into the fifth tube. Mix same and then withdraw 1 cc. and discard it. We now have in the 5 tubes dilutions of 1:100, 1:200, 1:400, 1:500, 1:1000, respectively, of the serum. To each tube, including the sixth which contains no serum, 1 cc. of antigen is added. This, of course, doubles the serum dilutions in the various tubes so that the same are now 1:200, 1:400, 1:800, 1:1000 and 1:2000 respectively. The sixth tube serves as an antigen control. It contains only salt solution and antigen and would indicate spontaneous agglutination of the antigen should such occur.

Agglutination with the first two dilutions may occur with serum from normal horses. If it occurs in as high a dilution as 1:800 the animal from which the serum was obtained should be considered suspicious and if clinical manifestations are in evidence, positive for glanders. Agglutination in a dilution of 1:1000 or over is considered a positive reaction.

As in the case with the complement-fixation test, mallein injections stimulate the production of agglutinins. This fact must be given considera-

tion in testing serum specimens from animals recently injected with mallein.

AGGLUTINATION TEST FOR BOVINE INFECTIOUS ABORTION

The antigen for the agglutination test for bovine infectious abortion is prepared in essentially the same manner as the glanders antigen. The growth from 48-hour old cultures is removed with sterile physiological saline solution, heated at 65°C. for one hour, diluted and preserved with 0.25 per cent formalin. One point to be observed in the preparation of bovine abortion antigen is not to have the final suspension too heavy. A heavy bacterial suspension of Alcaligines abortus often gives unsatisfactory results.

Serum Dilutions. The serum dilutions for the bovine infectious abortion test are much lower than those employed in the glanders test. The following dilutions can be made: 1:20, 1:40, 1:80, 1:160 and 1:320. To accomplish these dilutions set up a rack with 6 Wassermann size tubes and place 1 cc. of physiological saline solution in each tube. Make a basic dilution of the serum to be tested by adding one part of same to four parts of saline solution. Place 1 cc. of this dilution in the first tube, mix and carry 1 cc. over to the second tube. Mix this and withdraw 1 cc. and place it in the third tube. Proceed in the same manner through the fourth and fifth tubes, discarding the extra cubic centimeter of diluted serum in the fifth tube. The sixth tube serves as the antigen control and receives only the salt solution and antigen.

Complete agglutination in the 1:80 dilution and above is taken as evidence that the animal from which the serum was obtained is or has recently been infected with Alcaligines abortus. Agglutination in the 1:40 dilution may be considered suspicious.

AGGLUTINATION TEST FOR MALTA FEVER

To carry out an agglutination test for Malta fever in goats or sheep the instructions given above for the test for bovine infectious abortion apply. As a matter of fact, the same antigen can be employed. Agglutination in the 1:80 dilution or higher is considered positive.

AGGLUTINATION TEST FOR EQUINE INFECTIOUS ABORTION

The agglutination test for equine infectious abortion is carried out with the same dilutions as employed in the glanders test. Agglutination in a dilution of 1:1000 or more is considered positive, 1:800 suspicious.

In preparing the antigen, 24-hour old cultures of Salmonella abortus-equi are used rather than those grown for 48 hours. A better separation of the

organisms, giving a more even suspension, is obtained with 24-hour cultures. In other respects the antigen is prepared in the same manner as described for the glanders test.

AGGLUTINATION TEST FOR SALMONELLA PULLORUM INFECTIONS

Jones[4] and others have shown that excellent results can be obtained with the agglutination test in detecting hens harboring Salmonella pullorum in their ovaries.

In such cases agglutination with serum in a dilution of 1:100 or more is considered positive.

AGGLUTININ ABSORPTION TESTS

Agglutinin absorption tests are very valuable in the identification of various bacteria. If a quantity of bacteria is added to serum containing specific agglutinins the latter will be completely absorbed. This can be demonstrated by centrifugalizing the mixture, collecting the supernatant serum and using same in a regular agglutination test. Such serum would cause no agglutination of the specific bacteria. Where there is a close relationship between several bacteria the same is usually indicated by one organism partially absorbing agglutinins from serum specific for the other. Such partial absorption of agglutinins can be demonstrated by determining the agglutinating titre of the particular serum both before and after the absorption test.

The technic for carrying out agglutinin absorption tests is quite simple. The growth from a 24- to 48-hour slant agar culture of the particular organism is removed with sterile, physiological saline solution and placed in a graduated, conical bottom centrifuge tube of 15 cc. capacity. The bacterial suspension is centrifugalized at high speed for one-half hour. All of the supernatant fluid is then poured off and 10 parts of a 1:10 dilution of the serum added to the packed bacteria. With the aid of a platinum loop the bacterial mass is broken up and the mixture shaken and placed in the incubator for two hours. During the incubation period the tube should be shaken several times. Centrifugalize at high speed for one-half hour, draw off the supernatant serum and titrate the same against a known homologous agglutinating antigen, running a parallel control test with a similar dilution of the serum not subjected to the absorption test. Complete or partial absorption of agglutinins will be readily indicated by com-

[4] Jones, Rpt. N. Y. State Vet. College, Ithaca, N. Y., 1911–1912, p. 149.

paring the results obtained with the absorbed and control sera in the agglutination tests.

PRECIPITATION TESTS

A precipitating test consists of the bringing of a properly prepared antigen in contact with a suitable precipitating serum, a positive reaction being manifested by the formation of a distinct precipitate. In some tests the precipitating antigen and serum are mixed in small test tubes and after incubation the mixture examined for turbidity. In other tests the antigen is layered over the serum, a positive reaction being indicated by the formation of a milky ring at the zone of contact of the two fluids.

PRECIPITATION TEST FOR ANTHRAX

The precipitation test used in anthrax is frequently referred to as "Ascoli's test." As with all precipitating tests, a serum known to be suitable for the purpose must be available. Having such a serum, the test is carried out with an antigen prepared from the suspected material. To prepare the antigen place 1 or 2 grams of the suspected tissue, preferably spleen, in a test tube containing 5 or 6 cc. of sterile, physiological saline solution. Put the tube in boiling water and allow it to remain in same for 5 or 10 minutes. The mixture is allowed to cool when the fluid is filtered clear through asbestos wool and constitutes the antigen. One or 2 cc. of the antigen is drawn up in a pipette and carefully stratified over a few centimeters of the precipitating serum in a test tube, or preferably, a Uhlenhuth tube. If the tissue entering into the preparation of the antigen is from an animal which died of anthrax a milky zone, not unlike that noted in a positive reaction to Heller's ring test for albumin in urine, occurs at the point of contact of the two fluids. In most instances of positive reactions the ring appears in a very few minutes.

PRECIPITATION TEST FOR GLANDERS

The precipitation test for glanders is best carried out in accordance with the technic described by Pfeiler.[5] In this test the serum is the unknown and is tested against an antigen consisting of an extract of Pfeifferella mallei. Such antigen is prepared by washing off the growth from one or more 48-hour cultures of Pfeifferella mallei with a small quantity of sterile, distilled water and heating the suspension at 65°C. for one hour.

[5] Pfeiler, Arch. für Wissenschaftliche und Prakt., Tierheilkunde, Bd. 35, Hft. 4/5, June, 1909.

Such suspension is then further diluted with sterile, distilled water containing sufficient phenol to give the final product a concentration of 0.5 per cent of the preservative. The mixture should then be shaken in a shaking-machine for 1 or 2 hours on each of 5 or 6 consecutive days. At the end of this period the preparation is centrifugalized at high speed for one hour. The supernatant fluid is then drawn off and constitutes the antigen.

In carrying out the test a cubic centimeter of the suspected serum is placed in an Uhlenhuth tube or ordinary test tube and then with a pipette about 0.50 cc. of the antigen is carefully layered over the serum. The tube is left at room temperature and the results recorded within an hour. A positive reaction is denoted by the formation of a milky zone at the point where the serum and antigen are in contact.

PRECIPITATION TEST FOR SWINE ERYSIPELAS

Employing a suitable, swine erysipelas immune serum, precipitation reactions can be obtained with antigens consisting of tissue extracts prepared in the same manner as described for the anthrax test. A small amount of such antigen is stratified over the serum and results in the typical ring reaction if the tissue entering into the preparation of the antigen is from a case of swine erysipelas.

THE CONGLUTINATION TEST

Bordet and Gay observed that when red blood corpuscles are brought in contact with inactivated beef serum and fresh, active, horse serum there occurs an agglutination of the blood cells, followed by hemolysis of same. If the beef serum or horse serum alone is added to the blood cells practically no reaction occurs. Bordet and Gay explained the phenomenon on the ground that normal beef serum possesses a thermostable principle which serves to markedly increase the ability of fresh, normal horse serum to agglutinate and hemolize red blood corpuscles. They termed such principle "conglutinin." It was further found that if an antigen and inactivated serum containing specific antibodies for such antigen were brought in contact with the fresh, normal horse serum before the beef serum and blood cells were added, agglutination and hemolysis of the cells did not occur.

This phenomenon has been made use of in so-called "conglutination tests" for various diseases. In veterinary medicine such tests have been used, to a limited extent, for the diagnosis of glanders and dourine.

In carrying out the test 0.15 cc. of the inactivated, suspected serum is

placed in a test tube containing 2 cc. of physiological saline solution. Definite amounts of antigen (glanders antigen, for example) and fresh, normal horse serum are then added, the amounts of each being determined by titrations such as are employed in the complement-fixation test. The tube or tubes are then shaken and incubated at 37.5°C. for one-half hour if a water bath is employed or for one hour where a hot air incubator is used. After such period of incubation, definite amounts of titrated, inactivated beef serum and suspension of washed erythrocytes from a sheep are added. The mixtures are again incubated for one hour. A positive reaction is indicated by the cells settling to the bottom of the tube with no agglutination or hemolysis. In negative cases the red cells are rapidly agglutinated and then hemolyzed.

PART IX

HEMATOLOGY

CHAPTER XL

Clinical Blood Examinations

In the study of a number of diseases of man and the lower animals considerable information can frequently be obtained from blood examinations. Aside from bacteriological examinations for the demonstration of the etiological agents of various specific diseases as may occur in the circulation, the estimation of the number of red and white blood cells, differential leucocyte counts and hemoglobin determinations will often prove of material aid in making a diagnosis or prognosis.

THE ENUMERATION OF BLOOD CELLS

In estimating the number of red or white blood cells a Thoma hemacytometer or a modification of same is employed. The apparatus consists of a counting chamber and two diluting pipettes.

The original Thoma type of counting chamber consists of a heavy glass slide in the center of which is cemented a square of glass with a round hole in same. Into this round hole there is cemented a ruled disc having a diameter slightly less than that of the hole. Thus the disc occupies the center of the hole with a channel or moat around it. The disc is of such thickness that when a plano-parallel cover-glass is placed over the same it rests on the square glass leaving a space of exactly 0.1 mm. between the cover-glass and the ruled disc.

A counting chamber now employed on a rather large scale is that of Levy's. This consists of a heavy slide on the central portion of which is cemented a rectangular plate. This plate contains two ruled areas separated by a moat running transversely through the central part of the slide. On each side of this ruled plate a strip of glass, slightly thicker than the ruled plate, is cemented. The difference in thickness is such that when a plano-parallel cover-glass is placed over the ruled plate it rests on the two side pieces leaving a space exactly 0.1 mm. between the ruled plate and cover-glass. With this type of counting chamber a red cell count can be made on one half of the ruled plate and a white cell count on the other half. In other words, we have a double counting chamber which permits of both the red and white cell counts without stopping to clean up the apparatus between counts (Fig. 85). If desired, this same type of count-

ing chamber, i.e., with the rectangular plate, can be obtained with a single ruling.

Of several types of available rulings, that which is probably most used at the present time is Neubauer's (Fig. 86). With such ruling the area involved is divided into 9 large squares, each of which is exactly 1 mm. square. These 9 squares are each further subdivided into 16 medium-sized squares to facilitate counting. The central large square is still fur-

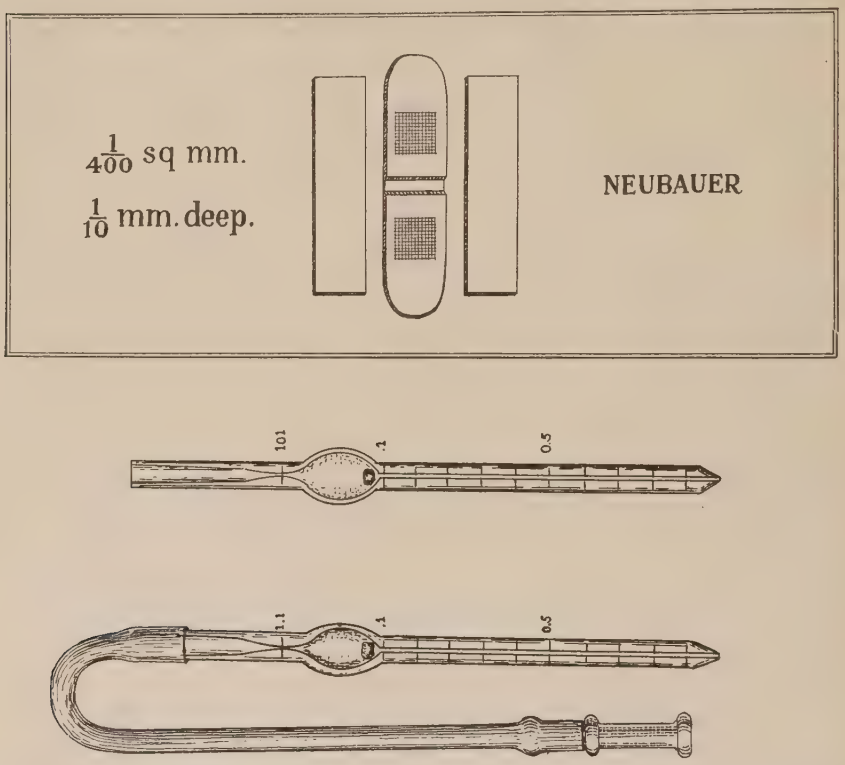


Fig. 85. Levy Counting Chamber and Pipettes for Red and White Cell Counts

ther divided into squares having an area of $\frac{1}{400}$ square millimeter. These latter are employed in counting red cells.

One of the two pipettes serves for the dilution and mixing of blood for red cell counts and the other for the same purpose in making white cell estimations. The graduated portion of the pipette for making red cell counts is calibrated so that the capacity of the pipette up to the "1" mark

is one-hundredth of that of the bulb when the pipette is filled to the "101" mark. The pipette for counting white cells is constructed to give a dilution of 10. Thus the capacity of the pipette up to the "1" mark is one-tenth that of the bulb when the pipette is filled to the "11" mark. Each pipette is supplied with a short piece of rubber tubing and a mouth-piece for drawing blood and diluting fluid into the pipette (Fig. 85).

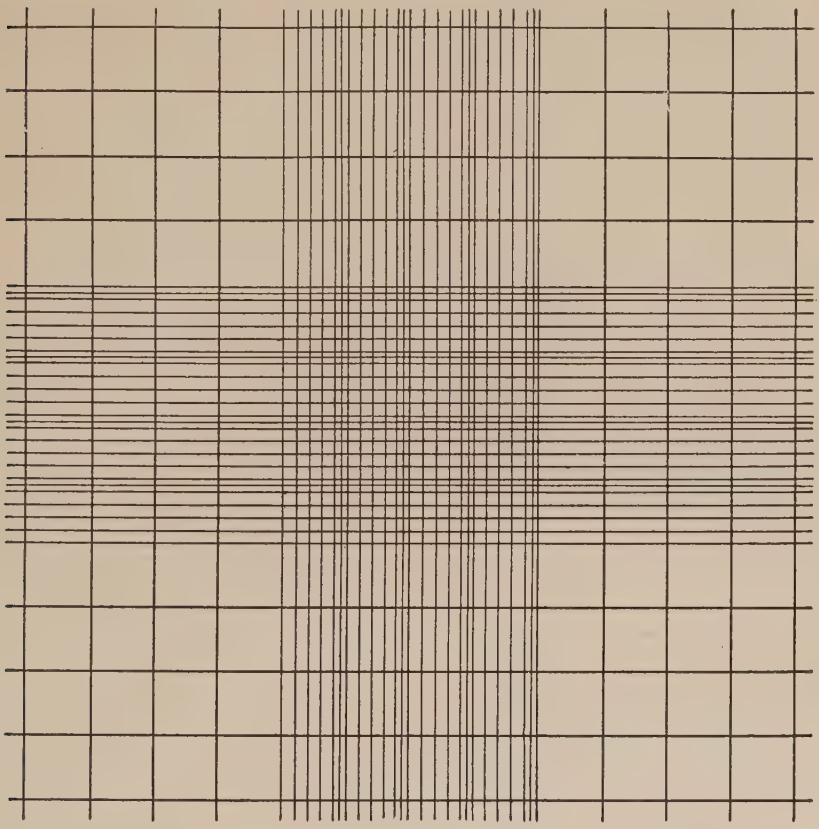


FIG. 86. NEUBAUER RULING

Diluting Fluids. Several types of diluting fluids have been recommended for use in diluting blood for red and white cell counts. The simplest diluting fluid for red cells, and one that is satisfactory, is ordinary physiological saline solution (0.85 per cent NaCl in distilled water). Two additional

agents which have been commonly used are Toisson's fluid and Hayem's solution. The formulae for these follow:

Toisson's Fluid

Sodium chloride	1.00 gram
Sodium sulphate	8.00 grams
Glycerin	30.00 cc.
Distilled water	160.00 cc.
Methyl violet	0.025 gram

Hayem's Solution

Mercuric chloride	0.50 gram
Sodium chloride	1.00 gram
Sodium sulphate	5.00 grams
Distilled water	200.00 cc.

Diluting fluids for white cell counts must be capable of laking the red blood corpuscles. The agent universally employed is a weak solution of acetic acid in distilled water. Some hematologists employ a 0.50 per cent solution of the acid while others use a 1 per cent dilution. In veterinary practice the author finds the stronger solution preferable. Some workers slightly tint their white cell diluting fluid with gentian violet. It is good policy to have one of the fluids tinged with gentian-violet but not both. This serves to readily differentiate them.

Technic for Red Cell Counts. A few drops of blood can be readily obtained from a horse or cow by piercing any one of the small vessels found about the facial region with a small, sharp lancet. Preparatory to taking the drop of blood a minute patch of hair should be shaved from over the area and the skin cleaned up with alcohol. In the case of the dog the blood can be obtained from the ear, or tail if he has one. With cats it is usually best to take the specimen from the tail.

After making the puncture allow a large drop of blood to collect and then touch the tip of the red cell pipette to it and carefully draw a column of blood up to the 0.5 mark. In cases of marked anemia it is sometimes advisable to make a lower dilution than usual, in which case the blood is drawn up to the 1 mark. After taking up the blood wipe the excess of same from around the end of the pipette and immediately draw up sufficient of the red cell diluting fluid to fill the pipette to the 101 mark. Catch the ends of the pipette between the thumb and second finger and shake the mixture for a minute or two. The small glass bead in the bulb facilitates mixing. A drop of fluid is then expelled to get rid of the small amount of straight diluting fluid in the capillary portion of the

pipette. To proceed with the count, place a small drop of the thoroughly mixed cell suspension on the ruled disc of the counting chamber. The size of the drop should be such that when the cover-slip is placed in position the space between it and the disc is filled without the fluid running over into the moat. The cover-glass is then carefully put in place, taking precautions to prevent getting air bubbles in the fluid. If the Levy type of apparatus is employed the procedure for filling the chamber is very simple. The cover-slip is placed in position first and then the tip of the pipette is touched against the ruled plate just under the edge of the cover-glass. As a result of capillary attraction, the suspension of cells will flow into the space between the cover-glass and ruled plate. After allowing two or three minutes for the cells to settle, place the slide on the stage of the microscope and with the 4 mm. objective count the red blood cells in 100 of the small squares ($\frac{1}{400}$ sq. mm.) in the central large square. Divide the figure obtained by 100 (simply point off 2 decimals) to determine the average number of cells for one small square. As the small squares are $\frac{1}{20}$ mm. square and as the distance between the ruled plate and the cover-glass is $\frac{1}{10}$ mm. the average number of cells observed in one small square represents the cellular content of $\frac{1}{4000}$ cu. mm. ($\frac{1}{20} \times \frac{1}{20} \times \frac{1}{10} = \frac{1}{4000}$) of the diluted blood. Then, to compute the number of red blood cells in 1 cu. mm. of undiluted blood, simply multiply the figure representing the average number of cells in one small square by 4000 and then by the amount of the dilution (200 or 100). For example, the total number of red cells in 100 small squares is 840, thus giving an average of 8.40 for each square; then $8.4 \times 4000 \times 200 = 6{,}720{,}000$ red blood cells per cubic millimeter of blood.

Technic for White Cell Counts. Cause the flow of a few drops of blood as indicated in the technic for red cell counts. Fill the capillary portion of the white cell pipette with blood up to the 0.5 mark (or in cases of leucopenia up to the 1 mark). Immediately draw up sufficient white cell diluting fluid to fill the pipette to the 11 mark. Thoroughly shake the mixture for several minutes. Microscopically it will be found that the white cells stand out as highly refractile bodies. Fill the counting chamber in the manner described for red cell counts. It should be remembered that the bore of the capillary portion of the white cell pipette is considerably larger than that of the red cell pipette and if held in an upright position the fluid will flow out freely. Thus, in filling the counting chamber, care must be taken not to flood same. After allowing a few minutes for the leucocytes to settle, count the number in 3 of the large squares (1 sq. mm. each). Three of the corner squares which are divided into 16 smaller squares by

single lines are usually selected. Determine the average number of leucocytes for one square by dividing by 3. As the distance between the ruled plate and cover-glass is only $\frac{1}{10}$ mm., the figure representing the average number of leucocytes in 1 large square (1 mm. square) must be multiplied by 10 to indicate the cellular content of 1 cu. mm. of the diluted blood. Then to determine the number of leucocytes in 1 cu. mm. of undiluted blood multiply by the dilution figure (20 or 10). For example, we find a total of 156 leucocytes in three of the 1 sq. mm. squares. The average for 1 square is 52. This is multiplied by 10 and then by the dilution figure 20. Thus, $52 \times 10 \times 20 = 10{,}400$ leucocytes per cubic millimeter.

Cleaning the Cell Counting Apparatus. Immediately after use the counting chamber, including cover-glass, should be well washed with soap and water, rinsed with clear water, and thoroughly dried.

The fluid in the pipettes should be expelled and a generous amount of clear water drawn through same. This process can be greatly facilitated by using a vacuum pump (Richards) attached to a water faucet. After washing thoroughly with water, alcohol is passed through the pipettes and this followed by ether. Following the ether a little air passed through the pipette will drive out any remaining vapor and leave the pipette perfectly dry.

ESTIMATION OF HEMOGLOBIN

A number of methods have been advocated for estimating the hemoglobin content of blood. It is not the purpose of this work to go into all of them. The following methods have found favor with a number of hematologists.

Dare's Method. In this method an apparatus known as Dare's Hemoglobinometer is employed. A drop of the patient's blood is allowed to enter the capillary space between the white and transparent glass plates. This is then placed in the instrument which is illuminated by means of either a candle or electric light. Looking through the eyepiece of the hemoglobinometer, the patient's blood is compared with a standard hemoglobin scale, the latter being moved by an adjusting disk until the colors match. The per cent hemoglobin which the patient's blood contains can then be read off directly from a scale on the instrument.

Sahli's Method. This is a rather recent method which seems to be rapidly gaining favor. The apparatus required is designated Sahli's Hemometer. It consists of a graduated tube and a color standard in a stand, and a measuring pipette. Until recently the color standard consisted of a

fluid. At the present time a hemoglobin standard made of solid glass rod can be obtained. It is claimed that the color of this glass rod will not fade.

In conducting the test, place into the tube, which is graduated from 10 to 140, sufficient of a N/10 solution of HCl to reach the mark 10. Draw into the capillary pipette 20 cu. mm. of the patient's blood. Carefully wipe any excess from around the tip of the pipette and discharge the contents into the tube containing the HCl. The blood should be all removed from the pipette by alternately drawing up and expelling the fluid. Shake the tube well and then let the solution stand 2 or 3 minutes. Next dilute the mixture in the graduated tube by adding water, drop by drop, until the color of the diluted blood matches the standard. The figure on the scale at the level of the meniscus of the fluid represents the percentage of hemoglobin.

Tallquist's Method. This is the simplest of all methods for the estimation of hemoglobin and probably for that reason has been used to a considerable extent in spite of the fact that it is not very accurate. The apparatus consists merely of a book containing filter paper squares and a color scale divided into 10 shades. A drop of the patient's blood is placed on a square of filter paper and this is placed under the color scale so that the dried blood drop shows through one of the holes in the scale. The area of each color shade has a hole in its center. By moving the filter paper under each shade, that which the dried drop of blood matches can be determined. The percentage of hemoglobin each shade represents is indicated. The ten shades run from 10 to 100. Where the color of a specimen of blood appears to be midway between two shades the average figure for the two is taken.

The Tallquist scale readily fades and admits of considerable error.

DIFFERENTIAL LEUCOCYTE COUNT

For differential leucocyte count several film preparations of the patient's blood are made on microscopic slides. Care must be taken to have a thin film with the cells evenly distributed. For this reason, several preparations should be invariably made. The films are stained after the method of Wright or Giemsa and examined with the microscope. A satisfactory preparation is selected and, with the oil immersion lens, one hundred (or better several hundred) leucocytes are counted. The different varieties are kept tract of by ruling off a sheet of paper into columns for the different types of white cells. Start at the upper left side of the film and determine the number of each type of leucocyte in the field. Note the number of

each type in the appropriate columns on the record sheet and then move the slide to the left just enough to take the old field entirely out of view. Now count the leucocytes in this field and note their numbers in the proper columns. Repeat the process, moving back and forth across the slide, until a total of 100 white cells has been counted. The number of each variety observed indicates the percentage of the particular type.

TABLE 8

Blood of Normal Animals

SPECIES	HEMOGLOBIN	RED BLOOD CELLS	WHITE BLOOD CELLS	POLYMORPHONUCLEARS	SMALL LYMPHOCYTES	LARGE LYMPHOCYTES	TRANSITIONALS	EOSINOPHILES	MAST
	per cent			*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Horse.	70–85	6,000,000–9,000,000	6,000– 9,000	60–65	25–30	2–5	1–2	3–4	0.5–1.0
Cow...	60–75	5,000,000–7,000,000	5,000– 8,000	40–45	48–52	4–6	2–4	5–7	0.5–1.0
Pig....	75–85	6,500,000–8,000,000	15,000–20,000	38–42	52–54	4–5	1–3	4–5	1 –2
Dog...	85–90	5,000,000–7,000,000	7,000–10,000	67–70	25–28	3–4	1–2	4–6	0–0.50
Cat...	65–75	5,000,000–6,000,000	10,000–12,000	58–60	35–38	2–4	2–3	4–5	0–0.50

NORMAL BLOOD OF ANIMALS

There is considerable variation in figures cited as normal for the red and white blood cells and hemoglobin content of the blood of some of the domestic animals. Table 8 indicates what might be considered a fair, normal average for the horse, cow, pig, dog and cat.

PART X

PREPARATION OF VETERINARY BIOLOGICAL PRODUCTS

CHAPTER XLI

The Production of Bacterins, Vaccines, Aggressins, Sera, Antitoxins, Tuberculin, Mallein, Etc.

The biological products treated of in this chapter may be conveniently divided into several groups. In the first group can be included those agents which have as their purpose the stimulation of an active reaction in the body of the animal injected, specific antibodies to combat or prevent disease resulting from such reaction. In other words, this group includes products which are designed to bring about active immunization of animals treated. In this class are the various bacterins, vaccines, aggressins, viruses and toxins.

The second group is composed of biologics which contain antibodies as a result of reaction on the part of the animal or animals used in the preparation of the product. Such agents do not stimulate the body of the recipient animal to an active reaction. Thus, the value of this type of product depends upon the antibodies it actually contains rather than upon ability to stimulate the production of specific antibodies. These are the biologics which produce passive immunity. This class consists of the various anti-bacterial sera, sera against several of the filterable viruses, and the antitoxins.

The third group is made up of those agents used in diagnostic allergic tests. In the presence of the disease for which they are specific, such agents stimulate a profound reaction, whereas in the absence of the disease no detectable disturbance occurs. This class includes the tuberculins, malleins, etc.

There has been considerable lack of uniformity in the use of the terms "bacterins" and "vaccines." Strictly speaking, a bacterin is any agent, used for the treatment or prevention of disease, which contains bacteria. Likewise, in a narrow sense, the term "vaccine" designates the cow-pox lymph preparation used for prophylactic immunization against small-pox. Both terms, however, have been employed in a much broader way and by many they are used synonymously. However, there is a tendency, with individuals primarily concerned with biological products, to employ the term "bacterin" to designate those products which are composed of *dead* bacteria and to reserve the term "vaccine" for such agents as contain *living* or *attenuated* organisms. The author is very much in favor of such rule for the use of these terms.

BACTERINS

A bacterin consists of a suspension of killed bacteria, usually in physiological saline solution. In some instances killed bouillon cultures are employed as bacterins. However, as a general rule, in the preparation of bacterins it is best to grow the organism or organisms on solid medium and prepare a saline suspension from the growth.

The procedure employed is essentially the same for all bacterins. The steps involved are as follows:

1. Isolate the organism or organisms in pure culture.
2. Propagate same on suitable medium just sufficiently long to obtain a satisfactory growth.
3. Examine culture for purity.
4. Remove growth with sterile, physiological saline solution.
5. Subject the suspension of bacteria to heat of a temperature no greater than that necessary to definitely insure destruction of the organisms.
6. Thoroughly shake the mixture to obtain a uniform suspension and then determine its bacterial content.
7. Dilute to desired bacterial content with sterile, physiological saline solution containing sufficient phenol or tricresol to make the final product contain 0.50 per cent phenol or 0.25 per cent tricresol.
8. Make aerobic and anaerobic cultural tests for sterility.
9. Fill in ampoules, phials or bottles.
10. Test a representative number of ampoules or bottles for sterility.
11. Inoculate experimental animals for safety test.

There are only a few points in connection with the above outline which need explanation. Young cultures are generally deemed preferable to old ones in the preparation of bacterins. As regards temperatures employed for the destruction of organisms entering into the preparation of a bacterin, it is a general rule that the less a biological product is heated, the better. However, the thermal death point of an organism and the minimum exposure period should be exceeded to such an extent as to definitely insure that the bacteria will be killed. Another general rule to follow in connection with the heating process, is never to add preservative to a biological product before heating. Thus, plain, sterile, physiological saline solution is used to remove the growth and the proper amount of preservative is added with the diluting fluid after heating.

Various methods have been employed for the standardization of bacterins and vaccines. For years the method almost universally employed was that developed by Wright. At the present time the Nephelometer method and the direct count method are, to a considerable extent, replacing the Wright method.

Wright Method. A capillary pipette, such as that used for opsonic index determination (Fig. 18), is marked with a wax pencil about 1 inch from the tip. Cleanse the finger, prick it and allow a good-sized drop of blood to collect. Draw up to the mark on the pipette 2 per cent sodium citrate in physiological saline solution, admit a small air bubble and draw up a similar quantity of blood. Another air bubble is admitted and a little of the bacterin to be standardized is drawn up to the mark. Now expel the contents of the pipette on a watch glass and accomplish a thorough mixture by alternately drawing up the material in the pipette and expelling it several times. Place a drop of the mixture on each of several scrupulously clean slides and with the aid of another slide spread the material out in thin films in the same manner that ordinary blood films are prepared. Dry in the air and stain according to Wright's method. Provide a reduced microscopic field by placing in the ocular of the microscope a disc of paper with a hole about $\frac{3}{8}$ inch in diameter in its center. With the oil immersion lens, count the number of red blood cells and bacteria in 50 fields and obtain the average figure of each for one field. Taking the figure 5,000,000 as representative of the number of red cells present in normal blood, the number of bacteria is obtained according to the following proportion: 5,000,000 : x : : the average number of red cells per field : average number of bacteria per field. For example, we find that the average number of red cells per field is 26 and that the bacteria average 52 per field thus, 5,000,000 : x : : 26 : 52 = 10,000,000 bacteria per cubic millimeter. As the bacterial content of bacterins and vaccines is commonly expressed to indicate the content of 1 cc., the figure obtained must then be multiplied by 1000.

Nephelometer Method. This method was developed by McFarland. It merely consists in comparing the density of bacterins or vaccines under examination with a series of standard suspensions of barium sulphate prepared to equal in density bacterial suspensions of known content.

The tubes composing the Nephelometer should be thoroughly shaken before use.

Direct Count Method. In this method a Helber counting chamber is employed and the bacterial suspension diluted and the individual organisms counted in a manner similar to that employed in estimating blood cells.

Plating Method. In standardizing bacterins some laboratories have employed the plating method for estimating bacterial content. This is accomplished by making dilutions of the suspension, just before the organisms are heated, definite amounts of such dilutions being inoculated into agar pour plates. After incubation the colonies are enumerated and the

bacterial content estimated in essentially the same manner as bacterial counts of milk are made.

This method is not very satisfactory.

VACCINES

As used in this text, the term "vaccine" designates a biological product containing living or attenuated organisms. Vaccines of importance in

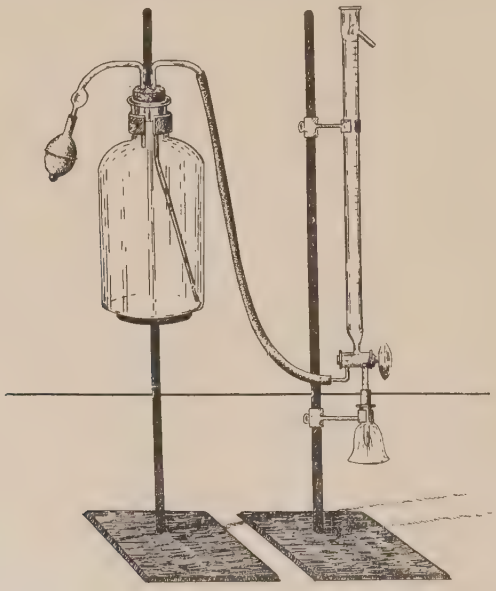


FIG. 87. APPARATUS FOR AMPOULING BACTERINS, VACCINES AND SERA

veterinary medicine include those for immunization against rabies, anthrax, symptomatic anthrax (blackleg), bovine infectious abortion, hemorrhagic septicemia, etc.

Rabies Vaccine (*Pasteur Method*). Healthy rabbits are inoculated subdurally with an emulsion of fixed virus in the manner described in the discussion of the virus of rabies (page 400). When completely paralyzed and about to die the rabbit is chloroformed. The carcass is then dipped in 3.5 per cent solution of liquor cresolis compound and tied on a clean dissecting board, belly down. Steps are then taken to remove the cord. It is important that the operation be conducted with all possible precautions to prevent contamination. The procedure should be carried out in a small,

"wet-down" room protected from draughts, and all instruments and equipment which will come in contact with the cord must, of course, be sterile.

With a sharp, sterile knife split the skin straight down the back and dissect it well away from the region of the vertebral column. With another sterile knife cut away the muscle tissue about the spine in the sacral and cervical regions. Then with sterile, bone-cutting forceps cut through the spinal column just back of the head and in the sacral region. The operator should now grasp the distal end of the spine with a pair of bone forceps and an assistant the upper end. A long, sterile, metal rod of such diameter that when the end is covered with a little cotton it can be readily forced into an adult rabbit's spinal canal, is employed by the operator for pushing out the cord. Just as it starts to emerge from the cervical end of the canal the assistant grasps it with a sterile pair of forceps, raising it as it is forced from the canal. Immediately the cord is out, the operator, using two pairs of sterile forceps, picks up one of several sterile loops of string previously prepared and kept in a Petri dish, slips the loop around the middle portion of the spinal cord, ties it and cuts off the spinal cord just above the knot. This lower half of the cord is immediately suspended in a cotton-plugged, salt-mouth bottle in the bottom of which are sticks of caustic potash. A second loop of string is tied to the top of the other half of the cord held by the assistant and this portion suspended in the same bottle as the first half. The bottle is properly labeled and the entire procedure repeated with such additional rabbits as are employed.

The bottles are then placed in a constant temperature chamber maintained at 22°C. and the different cords allowed to dry for periods of time varying from 2 to 8 days. As each cord completes its particular period of drying it can be used immediately for the preparation of vaccine or it may be cut up in pieces about 1 cm. long, placed in glycerin and stored in the ice-box. Dried cords thus preserved in glycerin should be used within ten days or two weeks.

The completed vaccine represents an emulsion of sections of the dried cord. Thus, we have seven different "strengths" of vaccine representing cords dried from 2 to 8 days, respectively.

If the vaccine is prepared for immediate use and not for shipment, the cord emulsions are best made with sterile, physiological saline solution. If the vaccine is prepared for shipment the nerve tissue is emulifised in 20 per cent glycerin. A piece of cord 1 cm. in length constitutes the usual amount for one dose for man and large dogs and for every such piece of cord 2 cc. of fluid are employed in making the emulsion. Horses receive 4 or 5 times the dosage for dogs.

Various schemes of treatment have been advocated and used in connection with the administration of rabies vaccine prepared according to Pasteur's method. When the Pasteur method is employed in veterinary medicine, treatment according to the scheme given in Table 9 is recommended.

Rabies Vaccine (Hogyes Method). In this method rabbits are inoculated with fixed virus and the spinal cords removed as described for the Pasteur

TABLE 9

DAY OF TREATMENT	DAYS CORDS DRIED	HORSES AND CATTLE		DOGS*	
		Amount of cord	Amount of fluid	Amount of cord	Amount of fluid
		cm.	*cc.*	*cm.*	*cc.*
1 (a.m.)	8	4	5	1	2
1 (p.m.)	7	4	5	1	2
2 (a.m.)	8	4	5	1	2
2 (p.m.)	7	4	5	1	2
3 (a.m.)	6	4	5	1	2
3 (p.m.)	5	4	5	1	2
4	5	4	5	1	2
5	4	4	5	1	2
6	4	4	5	1	2
7	3	4	5	1	2
8	2	4	5	1	2
9	4	4	5	1	2
10	4	4	5	1	2
11	3	4	5	1	2
12	2	4	5	1	2
13	4	4	5		
14	3	4	5		
15	2	4	5		
16	4	4	5		
17	3	4	5		
18	2	4	5		

* This dose for dogs should be reduced by one-fourth or one-half for small dogs.

method. One part of the nerve tissue is emulsified with 100 parts of sterile, physiological saline solution. For use dilutions of this emulsion are administered in increasing amounts so that the patient starts with a 1:1000 dilution and finishes with a 1:100.

When this type of vaccine is prepared for shipment the dilutions are made with physiological saline solution containing 20 per cent glycerin.

Rabies Vaccine (Harris' Method). Rabbits are inoculated subdurally

with fixed virus in the manner described under Pasteur's method of preparing rabies vaccine. When completely paralyzed they are chloroformed and both the spinal cord and brain removed under aseptic conditions. The tissue is then placed in sterile, crystallizing dishes and the membranes stripped off. This permits of a finer division of the tissue later on in the process. Cut the tissue up in small pieces with either a sterile scalpel or sharp scissors and make a paste of it by triturating it in a sterile mortar, adding a little sterile, physiological saline solution. There should then be in readiness a quantity of carbon dioxide snow, prepared by allowing carbon dioxide gas to rapidly pass through several thicknesses of sterile toweling, the snow collecting in the towel. This snow is gradually added to the paste as the triturating is continued. When solidification of the mass occurs the material should be immediately put through a sterile food-chopper which has been kept at a temperature below the freezing point. As the frozen tissue is run through the grinder a little additional carbon dioxide snow is added to it. The ground tissue is at once spread in a thin layer over the bottom of a large Petri dish, previously chilled, and the same immediately placed in a cold Scheibler desiccator containing H_2SO_4 or phosphoric anhydride. The desiccator is connected to an efficient vacuum pump and a vacuum of 2 mm. of mercury produced. The jar is then placed at a temperature of $-15°$ to $-18°$C. Complete desiccation ordinarily occurs within 48 hours.

The material is then pulverized in a sterile mortar in a dry atmosphere, placed in glass tubes and hermetically sealed. It should then be stored in a dark place at a temperature of $-10°$C. or less.

Aerobic and anaerobic cultural tests are carried out for the detection of possible contamination with pathogenic bacteria.

The minimal infective dose (M.I.D.) of each lot of desiccated virus is determined by injecting a series of rabbits with varying amounts of same. Such dose is usually from 0.002 to 0.006 mgm.

For horses and cattle the treatment should consist of a series of 15 injections starting with a dilution of 500 minimal infective doses of desiccated virus in saline solution. The amounts administered daily are gradually increased until 5000 M.I.D.'s are given. With dogs the treatment can be started with 100 M.I.D.'s and gradually increased to 1500.

For shipment the vaccine is sent out in the desiccated state, it being diluted just before use.

The advantages claimed for this type of vaccine are: better keeping qualities, making it possible to maintain a stock of same on hand; ability to more accurately gauge the dosage.

The preparation of the product is somewhat difficult because of the temperature requirements.

Rabies Vaccine (*Cumming Method*). Cumming found that when nerve tissue containing rabies virus was emulsified and dialyzed against distilled water, the material remaining in the dialyzing sac lost its ability to produce disease but was of value in immunizing against rabies.

The emulsified nerve tissue is placed in sterile, collodian sacs and dialyzed against distilled water for 24 hours. The emulsion remaining in the sac constitutes the vaccine and is administered in daily doses of 2 cc. (for man) over a period of 15 to 25 days. For dogs 1 cc. would be an average dose while for horses and cattle 6 cc. to 10 cc. can be administered.

Rabies Vaccine (*Method of Umeno and Doi*). The vaccine prepared by this method is that employed in the so-called "single injection method" of immunizing dogs against rabies. Of late this method has attracted considerable attention in the United States and is being employed on a rather large scale.

Rabbits are inoculated subdurally with fixed virus and when in the last stages of the disease they are chloroformed and the cord and brain removed with aseptic precautions. These tissues are then thoroughly triturated into a paste in a sterile mortar. To one part of the brain and cord substance there are slowly added 4 parts of phenolized glycerin-water. Such solution contains 60 per cent glycerin and 0.50 per cent phenol. After thorough mixture the emulsion is left at room temperature (18 to 22°C.) for two weeks or is placed in the refrigerator and left for a period of one month. Another method, employed by some laboratories to shorten the time, is to incubate it for 3 or 4 days.

After this period of standing the product is ready for use. The dose recommended by Umeno and Doi is 6 cc. per 10 kilograms body weight, puppies weighing less than 4.5 kgm., 3 cc. Schoening employed doses of 3 cc. for dogs weighing less than 20 pounds, 5 cc. for dogs between 20 and 40 pounds and 10 cc. for dogs over 40 pounds.

Where this vaccine is to be produced on a large scale, in place of using rabbits, horses of little value can be employed. When inoculated subdurally with fixed virus they develop the dumb form of rabies and, obviously, provide a large quantity of brain tissue.

Anthrax Vaccine (*Pasteur*). As indicated in the discussion of Bacillus anthracis, Pasteur found that when a virulent strain of the anthrax organism was grown for 24 days at a temperature of 42° to 43°C. it was reduced in virulence to such an extent that it would kill mice but not guinea pigs. After being thus reduced in virulence it was found that subsequent genera-

tions of such cultures could then be propagated at the usual incubator temperature of 37.5°C. without regaining their former virulence. Pasteur likewise demonstrated that when virulent cultures of the anthrax bacillus were grown at 42° to 43°C. for only 12 days they were attenuated to such an extent that they would kill guinea pigs but not rabbits.

Based on these findings Pasteur's method of immunization against anthrax was developed. He gave two injections of vaccine, allowing an interval of 12 days to elapse between injections. For the first injection he employed his "premier vaccin," which consisted of a bouillon culture of the anthrax strain reduced in virulence to the point where it was only pathogenic for mice. For the second dose the less attenuated culture was employed.

In attenuating cultures for the preparation of vaccine strains it will be found that the periods of 24 and 12 days, given as the time of growth at the high temperature for the production of the weaker and stronger cultures, respectively, are only approximate. Thus, in order to obtain cultures of the desired attenuation, 25 or 30 bouillon cultures should be prepared from each of 2 or 3 strains of virulent Bacillus anthracis. These cultures are then placed in an incubator maintained at 42° to 43°C. Starting at about the eighth day a culture of each strain is removed from the incubator daily and rabbits and guinea pigs inoculated until a strain capable of killing guinea pigs but not rabbits is obtained. Then the incubation period is continued and the representative cultures removed daily are inoculated into guinea pigs and mice, the procedure being continued until a culture which will kill mice but not guinea pigs is obtained.

Animal inoculation tests with newly produced vaccine strains should be repeated several times to demonstrate beyond all doubt that the cultures have been properly attenuated.

Pasteur anthrax vaccine proper is merely a bouillon culture of such attenuated strains of Bacillus anthracis. The vaccine referred to commercially as the "No. 1" is, obviously, the weaker, the "No. 2," the stronger. One cubic centimeter of the No. 1 vaccine is administered subcutaneously, followed in 10 days with a like amount of the No. 2 vaccine.

As sheep are highly susceptible to anthrax the second injection usually consists of a vaccine which is mid-way between No. 1 and No. 2 in virulence. If desired, vaccine of No. 2 virulence can then be given as a third dose after the elapse of a second 10-day period.

It has been found that anthrax vaccine prepared according to the original Pasteur method deteriorates rather rapidly, the product apparently undergoing autolysis. For this reason, in place of using bouillon cultures of the organism, it is a better procedure to grow the vaccine strains on agar and

prepare the vaccine in the form of a suspension of the living organisms in physiological saline solution.

Obviously, all lots of anthrax vaccine should be tested bacteriologically for purity and suitable laboratory animals inoculated to check the degree of virulence.

Anthrax Spore Vaccine (for Use in Simultaneous Method of Immunization). Anthrax spore vaccine, as here described, is prepared from a vaccine strain of Bacillus anthracis of the virulence of the Pasteur No. 2 type, i.e., it is virulent for mice and guinea pigs but not for rabbits. This vaccine is employed in the simultaneous method of immunization against anthrax, being administered with anti-anthrax serum.

The vaccine culture is grown for 5 to 7 days on peptone-free agar. At the expiration of such period the cultures will be found to consist largely of spores. The growth is then removed with sterile, physiological saline solution and an even suspension obtained by thorough shaking. The bacterial content of this suspension is determined (usually by the Nephelometer or plating method) and the product diluted to contain 10,000,000 organisms per cubic centimeter. This constitutes the vaccine. For use 1 cc. of same is administered at the same time that 10 cc. of anti-anthrax serum are given. The dose of vaccine for sheep is usually reduced to one-half of the amount given horses and cattle.

Symptomatic Anthrax (Blackleg) Vaccine. (Kitt's Modification of the Method of Arloing, Cornevin and Thomas). One or more healthy yearlings are inoculated intramuscularly in the rump region with an emulsion of dried, blackleg muscle tissue held over from a previous case of the disease. Inoculated animals will ordinarily succumb within 48 hours.

Immediately following death of the animal the skin is dissected from the involved region and all of the affected musculature carefully removed. As much of the fat and fascia as can be removed is trimmed away and the affected muscle tissue cut in long, thin strips. A small metal hook is placed in the end of each piece of tissue and the strips hung on wires stretched in a drying chamber.

After the tissue has become thoroughly dry, as determined by breaking several of the brittle strips, it is put through a spice mill several times to reduce it to a coarse powder. Such powder is then mixed with sufficient sterile water to give it a dough-like consistency. This mass is then distributed in thin layers in small, metal pans about $4\frac{1}{2}$ by $2\frac{1}{2}$ by $\frac{1}{8}$ inches. These pans are then placed on shelves in a hot air attenuating oven maintained at a temperature of 97°C. A pan of water is put in the bottom of the oven to keep the atmosphere saturated with moisture. The product is

heated for 6 hours, the pan of water in the oven being removed about one-half hour before completion of the heating process. It will then be found that the ground tissue has taken the form of dry, thin scales which tend to curl away from the small pans.

The next step is to put these scales through a special mill which will reduce them to a fine powder. Such powder constitutes the vaccine. A representative sample of same is emulsified in a few cubic centimeters of saline solution and several guinea pigs inoculated intramuscularly to demonstrate that the product is safe for use. A mild reaction on the part of the guinea pigs, manifested by temperature elevation and slight local swelling, is desirable.

The vaccine is supplied in powder form and for use is administered in doses of 0.10 gram emulsified in a little physiological saline solution or sterile water.

This type of vaccine has also been prepared in pellet form for administration with a special type of injector.

For many years blackleg vaccine was prepared as outlined above and distributed by the Bureau of Animal Industry of the United States Department of Agriculture and proved a potent factor in the control of the disease in the United States.

Hemorrhagic Septicemia Vaccine. Hemorrhagic septicemia vaccines, particularly for the vaccination of cattle, have been prepared from cultures attenuated by various means. In some instances the attenuation has been brought about by growing a strain of the organism at high temperatures (42° to 43°C.). In other cases cultures have been used which became attenuated as a result of many generations' growth on artificial media.

Bovine Infectious Abortion Vaccines. Of the vast amount of work done with biological products in bovine infectious abortion, the results, as previously indicated, have been very discouraging. There is some evidence, however, to indicate that vaccines consisting of live cultures of Alcaligines abortus may, at times, prove of value in the control of the disease. Such vaccines merely consist of a heavy suspension of Alcaligines abortus in physiological saline solution.

A number of investigators question the advisability of administering living organisms of this type on a wholesale scale.

AGGRESSINS

It has previously been indicated that the term "aggressin" was first used by Bail to designate a principle which he conceived various bacteria capable of producing and which functions by antagonizing the body factors

detrimental to the existence of the bacteria. It has also been pointed out that a number of investigators consider Bail's aggressins as being nothing more than endotoxins. However, irrespective of what the exact nature of such principle may be, it has been definitely proved that products of certain bacterial processes, when freed of the living bacteria which they contain and then injected into susceptible animals together with a slightly virulent strain of the particular organism, the virulence of the latter is greatly enhanced. It has been further demonstrated that the injection of the germ-free exudate alone will, in several diseases at least, immunize the animals, thus injected, against infection with the organism which caused the disease process from which the exudate was collected. This is explained on the ground that anti-aggressins are produced by the animal as a result of the reaction which the aggressin-containing exudate caused. On subsequent infection such anti-aggressins will promptly neutralize the aggressins produced by the invading bacteria and thus permit of their destruction by the defense forces of the body.

In the United States several so-called "aggressins" have attracted considerable attention in veterinary medicine. The first to gain favor was that prepared for use against symptomatic anthrax (blackleg). Then a product for immunization against hemorrhagic septicemia came to the front. Both of these products have been subjected to severe tests and have stood up well. Insofar as symptomatic anthrax is concerned the author has tested various products for immunization against such disease and has no hesitancy in placing the so-called "blackleg natural aggressin" at the top of the list. I am firmly convinced that as regards its efficacy as a prophylactic agent, this product is in the class with tetanus antitoxin and small-pox vaccine.

As this is being written, reports of the successful use of an anthrax aggressin in the prevention of such disease have been published by Salsbery.[1]

Symptomatic Anthrax (Blackleg) Aggressin. According to the method of Franklin and Haslam, young cattle are injected with 1 gram of dried muscle tissue or 20 to 30 cc. of culture. Immediately following death the fluids and tissue from the affected area are carefully collected. The affected tissue is finely ground in a meat-chopper, added to the fluid collected from the region of the disease process and the mass frozen. The mass is then allowed to thaw and the fluid collected and put through a Berkefeld filter of "N" porosity. Preservative is then added in the form of 1 per cent chloroform or 0.50 per cent phenol, the former being preferable.

This process can be somewhat simplified as follows: Inoculate the animals as indicated above and harvest the affected tissue and fluids. Grind

[1] Salsbery, Jour. A. V. M. A., n.s., vol. 21, no. 6, March, 1926.

the tissue thoroughly and by means of an efficient press extract all of the fluid possible from same. Add this to the fluid collected direct from the involved area. Then add preservative and allow the mixture to stand for a while. A large part of the suspended material will settle out. The supernatant fluid is then drawn off and put through a paper pulp filter, using a Buchner funnel. Then the lower portion of the tissue extract is filtered through paper pulp, this second filtrate being kept separate from the first as it filters through the Berkefeld more slowly. The two lots of filtrates are now put through a Berkefeld filter of "N" porosity, that representing the supernatant fluid being passed through the filter first.

The filtered product constitutes the aggressin. It is tested culturally and subjected to guinea pig inoculation tests. For use it is administered in doses of 5 cc.

Hemorrhagic Septicemia Aggressin. In July 1924 Gochenour[2] published the results of investigations which have led to the development of an aggressin for immunization against hemorrhagic septicemia. He isolated from an outbreak of hemorrhagic septicemia among American buffalo (bison) an exceedingly virulent strain of the Pasteurella organism. Inoculation of large animals with this strain rapidly caused death and from the edematous fluids of the subcutaneous tissues he was able to prepare an aggressin which proved highly valuable as an immunizing agent. Since his report hemorrhagic septicemia aggressin has become an important factor in the control of the disease.

For the production of the product either cattle or horses may be employed. The culture is injected subcutaneously in the neck region. Within 18 to 24 hours a pronounced swelling will be noted about the site of injection with the edema extending down into the brisket region. Death usually ensues within 24 to 48 hours. Post-mortem examination will reveal a vast amount of subcutaneous and intramuscular edema. This edematous material is carefully collected and rendered sterile by first filtering through paper pulp and then through a Berkefeld filter. Preservative is added in the form of 0.50 per cent phenol or 1 per cent chloroform.

The product is tested for sterility by cultural and animal inoculation tests.

For use hemorrhagic septicemia aggressin is administered to cattle and horses in doses of 5 cc. Calves, sheep and hogs are given doses of 2 cc.

CULTURE FILTRATES (SO-CALLED "ARTIFICIAL AGGRESSINS")

In work with agents for immunization against symptomatic anthrax (blackleg) it has been found that Berkefeld filtrates of cultures of Clostrid-

[2] Gochenour, Jour. A. V. M. A., n.s., vol. 18, no. 4, July, 1924.

ium chauvei, especially cultures in Martin's peptone solution to which ground beef has been added, are of value for immunizing animals against the disease.

Such product is not as efficacious as the so-called "natural aggressin" prepared from extracts of affected tissue.

SERA AND ANTITOXINS

Various anti-bacterial sera are produced for the treatment and prevention of infectious diseases. Some such sera are of established value while the results obtained with others have been discouraging. Sera of considerable value have also been produced against several of the filterable viruses—hog cholera, rinderpest, etc. Then, we have the antitoxic sera specific for the toxin of the tetanus, diphtheria and botulism organisms.

Antibacterial sera are prepared by injecting horses or other suitable animals with gradually increasing amounts of cultures of the organism or organisms they are to be hyperimmunized against. Preparatory to injecting the animals with living organisms they are usually first given immunizing treatment consisting of the injections of killed or attenuated bacteria.

Where animals are employed for antitoxin production, they are given gradually increasing amounts of carefully standardized toxin.

Anti-anthrax Serum. For the production of anti-anthrax serum horses, mules or cattle are usually employed. Where serum is administered to cattle in anthrax districts at regular intervals cases of anaphylactic reactions frequently occur when the anti-anthrax serum used is from horses. For this reason biological firms prepare anti-anthrax serum for both bovine and equine use.

Animals used for the production of the serum are first immunized by either the simultaneous or Pasteur method. Two weeks after such treatment each serum animal is given twice the amount of vaccine it received in the immunization treatment. This is usually 2 cc. of Pasteur No. 2 vaccine or 2 cc. of spore vaccine of Pasteur No. 2 virulence. After the elapse of one week 4 cc. of the vaccine are given and this followed after a further period of one week with 10 cc. of vaccine. One week following such injection the first dose of living, virulent culture is given. The amount inoculated is one-hundredth of the amount of growth from a 24-hour agar culture, that can be picked up in the loop portion of a standard platinum loop. Injections of virulent culture are then repeated at weekly intervals. For the second dose 1 loopful of culture is administered. The process is then continued as indicated at top of page 477.

INJECTION	AMOUNT OF CULTURE
1	0.01 loopful
2	1.0 loopful
3	5.0 loopfuls
4	10.0 loopfuls
5	¼ growth from 1 agar slant
6	½ growth from 1 agar slant
7	1 agar slant culture
8	2 agar slant cultures
9	3 agar slant cultures
10	5 agar slant cultures
11	1 Kolle flask culture
12	2 Kolle flask cultures
13	4 Kolle flask cultures
14	6 Kolle flask cultures
15	8 Kolle flask cultures
16	10 Kolle flask cultures

The injections may be given either subcutaneously or intravenously. When the larger amounts are given subcutaneously the culture suspension in physiological saline solution should be distributed over several areas and not inoculated in one place. In removing the growth from the agar, no more saline solution than necessary should be employed. If the injections are given intravenously the material must be thoroughly shaken and injected very slowly. The two larger amounts of culture are omitted when the intravenous method is employed. In case of severe reaction an injection due is delayed until such reaction subsides.

Two weeks after the last injection the animal is bled from the jugular vein, the serum separated and filtered through paper pulp or asbestos wool and then through a Berkefeld filter of "N" porosity. This constitutes the finished product which is then subjected to cultural and animal inoculation tests.

Anti-hog-cholera Serum. Anti-hog-cholera serum is obtained from hogs hyperimmunized against the filterable virus of the disease. As the etiological factor of hog cholera cannot be grown artificially, the virus employed for the hyperimmunization of hogs must be propagated in the body of susceptible pigs.

For the production of virus, pigs weighing around 100 pounds are desirable. They are inoculated and their temperatures taken twice a day. They usually develop evidence of disease in five to ten days. At the height of the disease, as indicated by temperature elevation, the virus pigs are bled

to death. The collected blood is defibrinated and employed for hyperimmunization and also for use in practice in connection with the simultaneous method of immunization against hog cholera.

Large hogs selected for hyperimmunization are first immunized by the simultaneous method and are then injected with virus blood alone. For each pound of body weight 5 cc. of virus blood are injected intravenously, using the ear vein. After a period of 10 days such hogs are bled for serum. The bleeding is ordinarily done from the tail, cutting off the end of same and employing a vacuum apparatus. Precautions are taken, of course, to properly clean and disinfect the tail and sterile equipment is used. The amount of blood taken is 5 cc. per pound weight of hog. The blood is usually collected in citrate solution to prevent clotting. After a lapse of one week the pig is bled a second time. Then after another week it is bled to death from the heart.

In collecting the blood in sodium citrate, 2 per cent of a saturated solution is employed. The cells are separated from the serum through centrifugalization. This is facilitated if the red blood cells are first agglutinated with bean extract as described by Dorset and Henley. A further amount of sodium citrate is added to the clear plasma to raise the content to 4 per cent. The fluid is then heated at 60°C. for one hour to precipitate the fibrin. The heating process is also a safeguard against the transmission of filterable virus diseases, such as aphthous fever, by hog cholera serum. After precipitation of the fibrin 0.50 per cent phenol is added (using 1 per cent of a 50 per cent solution of phenol in ether—this will not precipitate the serum proteins) and the serum Berkefeld filtered. Cultural tests for sterility are conducted and potency tests carried out on hogs.

In place of preparing a clear serum, such as described above, the blood from hyperimmune hogs can be simply collected, defibrinated and preserved with phenol and is ready for use. Such type of product was universally employed for a number of years but is rapidly disappearing.

A concentrated hog cholera serum can be prepared by isolating the pseudo-globulin of the serum by fractional precipitation with ammonium sulphate, the ammonium salt being subsequently removed by dialysis. This process is similar to that employed in the refinement of tetanus and diphtheria antitoxins.

For immunization against hog cholera both the serum alone or serum and virus (simultaneous) methods are employed. The serum alone confers a passive immunity of short duration. When serum and a small amount of virus are administered simultaneously an active immunity of considerable duration results.

Anti-rinderpest Serum. An anti-rinderpest serum can be prepared by hyperimmunizing recovered or immunized animals, through a series of injections of large quantities of blood from animals affected with rinderpest. The virus blood is taken from the affected animal at the peak of the disease. It is collected in a little sodium citrate solution or it can be defibrinated. Serum animals are injected subcutaneously, starting with 100 cc. of virulent blood and gradually increasing the amount administered until several liters are given. The injections are made at 5-day intervals. Some investigators give the injections intraperitoneally.

Anti-rinderpest serum is used both alone and in conjunction with virus (simultaneous method) for immunization against rinderpest.

Other Sera. In addition to the sera referred to above, anti-bacterial sera are prepared and marketed for a number of diseases met with in veterinary medicine. Thus, we find on the market sera for use in staphylococcic and streptococcic infections. For Escherichia coli infections, such as dysenteria neonatorum and pyo-septicemia of sucklings, a serum is obtainable. Equine influenza, canine distemper, hemorrhagic septicemia, symptomatic anthrax and swine erysipelas are other diseases for which sera are prepared.

Such anti-bacterial sera are produced by injecting horses, cattle or other suitable animals with increasing amounts of cultures of organisms concerned in the particular disease for which the serum is prepared. In some instances, where the etiological factor is a filterable virus or is unknown, the organisms entering into the preparation of the serum are only secondary factors.

Some of these anti-bacterial sera are of distinct value while the merits of others are questionable.

Tetanus Antitoxin. Tetanus antitoxin represents one of the best biological products we have at our disposal. Ability to prevent tetanus in exposed individuals, including the lower animals, by the timely administration of tetanus antitoxin has become such an established fact that it admits of no question. Unfortunately, however, this biologic is far less efficient in the treatment of the disease after symptoms have appeared.

Tetanus antitoxin is prepared by injecting horses, subcutaneously, with gradually increasing amounts of tetanus toxin.

The toxin is prepared by growing Clostridium tetani in flasks of dextrose-veal bouillon. The medium is filled up to the neck of the flask to reduce the amount of air space. Liquid petrolatum can then be layered over the small surface exposed. Just before inoculation the flasks of bouillon are heated to drive off any oxygen the medium might contain and after cooling down to 50°C. they are inoculated with a culture of Clostridium tetani. The cultures are then incubated for 15 days at 37.5°C. If incubated for more than 20 days, an appreciable loss of potency occurs.

The cultures are then filtered through sterile, asbestos wool or paper pulp in a Buchner funnel and then through a Berkefeld filter of "N" porosity. To the toxic filtrate phenol is added to the extent of 0.50 per cent, using a 5 per cent solution. It is then standardized by inoculating graduated quantities into a series of several guinea pigs each weighing approximately 350 grams. Dilutions of 1:10,000, 1:15,000, 1:20,000 and 1:25,000 of the toxin can be made and such dilutions administered in 1 cc. subcutaneously, to guinea pigs.

Healthy horses to be used for antitoxin production are first given 2000 to 3000 units of tetanus antitoxin and in a day or two are then started on a small dose of toxin, usually 10 to 20 guinea pig minimal lethal doses (M.L.D.). The injections are made subcutaneously at intervals of 3 days, the amounts of toxin gradually being increased until the animal is receiving 750 to 1000 cc. of potent, toxic culture filtrate.

Several times towards the end of the hyperimmunizing process a small amount of blood can be obtained from the horse, the serum separated and tested for its antitoxin content.

Ten days after the last injection of toxin the horse can be bled for antitoxin production.

Whole serum can be employed as antitoxin, or better, it can be concentrated by separating from the serum the pseudo-globulin, which contains the antitoxin, by the method first described by Gibson and Banzhaf of the New York Board of Health Laboratories. The method involves the fractional precipitation of the globulins with ammonium sulphate, such salt later being eliminated from the globulin by dialysis.

The serum or globulin solution is phenolized to the extent of 0.50 or 0.25 per cent tricresol is added and the fluid Berkefeld filtered.

The product must now be standardized. The unit of antioxin is determined by inoculating a series of guinea pigs, each weighing approximately 350 grams, with mixtures containing a definite amount of toxin plus variable amounts of the antitoxin under test. The various mixtures of toxin and antitoxin are made, allowed to stand at room temperature for one-half hour and then injected into the guinea pigs subcutaneously.

In order that the unit of antitoxin may be standard the Hygienic Laboratory of the United States Public Health Service supplies to laboratories manufacturing antitoxin a standard tetanus toxin against which antitoxins are tested. The *unit* of tetanus antitoxin is defined as 10 times the smallest amount of antitoxin which will protect a 350-gram guinea pig for 96 hours against the *official test dose* of the standard toxin furnished by the Hygienic Laboratory, United States Public Health Service. The official test dose

contains 100 M.L.D.'s for a 350-gram guinea pig, hence, a unit of tetanus antitoxin is actually 1000 times the smallest amount necessary to protect a 350-gram guinea pig against one M.L.D. of toxin, for 96 hours.

Botulism Antitoxin. Botulism antitoxin is prepared essentially in the same way as tetanus antitoxin, horses being given gradually increasing amounts of toxin.

However, it must be remembered that there are two types ("A" and "B") of Clostridium botulinum and that the antitoxin of one type will not protect against the antitoxin of the other. Thus, two types of antitoxin must be prepared and these can be pooled to form a polyvalent product. In producing botulism antitoxin it has been found impossible to prepare a satisfactory polyvalent antitoxin with a single horse. One animal is therefore employed for one type of the antitoxin and another for the second type and the serum of the two horses later pooled.

TUBERCULIN AND MALLEIN

Tuberculin and mallein both occupy a very important place in veterinary medicine. In measures for the control and eradication of tuberculosis in cattle and glanders in horses, these two biological products are indispensable agents.

Tuberculin (O.T.). Cultures of Mycobacterium tuberculosis are grown for a period of eight weeks upon the following medium:

Beef broth	1000 cc.
Peptone	10 grams
Glycerin	50 cc.
Potassium phosphate, monobasic	5 grams

Adjust the reaction to pH 7.2 and sterilize fractionally in the Arnold sterilizer.

Such cultures are then sterilized by one hour exposure in the Arnold sterilizer and are then poured into large evaporating dishes and concentrated to one-tenth of their original volume. This concentrated material is then allowed to settle, is filtered through paper and then through a Berkefeld filter of "N" porosity, 0.50 per cent phenol being added just prior to the Berkefeld filtration. This constitutes Koch's Old Tuberculin (O.T.).

Tuberculin (Subcutaneous). Subcutaneous tuberculin is merely a dilution of Koch's old tuberculin. The extent of the dilution varies with the product prepared by different laboratories. Ordinarily it is diluted with 3 to 6 parts of sterile, phenolized saline solution. Irrespective of the dilution the dosage for cattle should be such that each animal receives 0.50 gram of Koch's old tuberculin.

Tuberculin (Intradermal). Intradermal tuberculin can be prepared according to the same procedure outlined for Koch's old tuberculin with the exception that the concentration is carried out to only one-fifth of the original volume of culture. If old tuberculin (Koch) is on hand, a product for intradermal use can be prepared by simply diluting such tuberculin with an equal volume of sterile, phenolized saline solution.

Tuberculin (Ophthalmic). Ophthalmic tuberculin is prepared for use both as a liquid and in the form of so-called "ophthalmic tuberculin discs." The procedure which is followed by the United States Bureau of Animal Industry and the Army Medical School in preparing this type of tuberculin is as follows:

Cultures of Mycobacterium tuberculosis are grown on the bouillon medium described for the preparation of Koch's old tuberculin for eight weeks and concentrated to one-fourth of their original volume. The concentrated material is then allowed to settle or is filtered through paper. Place 200 cc. of this concentrated tuberculin in a balloon flask of 2000 cc. capacity. With constant shaking allow 2000 cc. of acetone to enter the flask a few drops at a time. This is a tedious portion of the procedure and can be facilitated by having the flask containing the tuberculin in a shaking cradle and an arrangement made for the acetone to enter the flask, a few drops at a time, through a small rubber hose connected with a bottle containing the 2 liters of acetone. This mixture is allowed to stand for 48 hours. At the expiration of such period as much of the acetone as possible is decanted off of the precipitate and the last traces driven off with the aid of compressed air. The precipitate is dissolved in 200 cc. of sterile, distilled water. To this then add 470 cc. of 95 per cent alcohol, agitating the mixture as the alcohol is poured in. Let this stand for 24 hours and then collect all of the fluid, discarding the precipitate. The last of the fluid siphoned off usually has to be filtered to render it free of precipitate. The alcoholic solution is then evaporated over a steam bath until all traces of alcohol are driven off. The material remaining in the evaporating dish is then brought up to a volume of 200 cc. of sterile, distilled water. This mixture is again precipitated with 2000 cc. of acetone as previously described. Let the mixture stand for 48 hours, decant off as much of the acetone as possible and drive off remaining traces with compressed air. The precipitate is then taken up with a small volume of water and placed into a small evaporating dish on a steam bath. It is allowed to remain there until it becomes pasty. The consistency of this paste should be such that when 20 grams of lactose are added a putty-like mass results. This is molded in a ball, put in a small evaporating dish, same placed in a desiccator containing

sulphuric acid and a vacuum drawn on same until the tuberculin mass raises in the form of a light, tan colored "biscuit." In this condition the mass will thoroughly dry within 24 hours. It is then ground in a thoroughly dry mortar and the powder put through an 80-mesh sieve. Such powder is immediately placed in dry bottles, tightly corked and used for the preparation of discs. It is important that the powder be handled in an atmosphere of low humidity as it is very hydroscopic.

The discs are prepared by adding about 1 cc. of absolute alcohol containing 2 or 3 drops of water to every 3 grams of powder. Such material is molded into discs through the use of a tablet mold containing holes $\frac{3}{16}$ inch deep by $\frac{5}{16}$ inch diameter. These tablets are immediately put up in small phials containing a bit of cotton, tightly corked and sealed with paraffin.

In making liquid ophthalmic tuberculin the above procedure is followed up to the point where the lactose would be added. In place of adding the lactose the concentrated tuberculin is taken up in 100 cc. of water containing 37.5 cc. of glycerin.

Mallein (*Intradermal*). For the production of intradermal mallein cultures of Pfeifferella mallei are grown for eight weeks in 3 per cent glycerin broth of pH 6.8 for 8 weeks or longer. These cultures are then sterilized by subjection to one hour's exposure to live steam in the Arnold sterilizer. The individual cultures are then poured into bottles of from 3 to 10 liters capacity, depending upon the number of cultures, again sterilized, and allowed to stand in the refrigerator for one or two days to permit settling of most of the bacteria. The supernatent fluid is then drawn off and concentrated to one-tenth of its original volume. This is then filtered through paper, or it may be put up in bottles, sterilized fractionally, preserved with 0.50 per cent phenol and stored as crude mallein for the future preparation of either ophthalmic or intradermal mallein. Intradermal mallein is made by diluting one part of such crude mallein with three parts of sterile, physiological saline solution containing 0.50 per cent phenol, and Berkefeld filtering.

Mallein (*Ophthalmic*). Ophthalmic mallein is simply crude mallein, prepared as above described, and clarified either by allowing it to stand for a number of weeks to permit the settling of suspended material or by Berkefeld filtering it. Because of the sirupy consistency of the product Berkefeld filtration is very slow.

Mallein (*Subcutaneous*). Subcutaneous mallein, in the United States, is very little used at the present time. This product consists of a dilution of crude mallein, the extent of the dilution varying somewhat with different manufacturers. Irrespective of the dilution the dosage should be such that adult horses receive one to two grams of the crude mallein.

PART XI

BACTERIOLOGICAL EXAMINATION OF MILK AND WATER

CHAPTER XLII

Examination of Milk. Certified Milk. Pasteurization. Examination of Water

Proper and adequate supervision of a community's milk supply is an important function of public health service. Such supervision involves detailed inspections of dairy farms, their equipment, methods, water supply, health of cattle, health of employees, etc. These factors are highly important and are made the subject of a regular course in milk and dairy hygiene found in the curriculum of all accredited veterinary colleges.

Another important phase of milk-supply control is the routine bacteriological examination of milk. Such examinations are made principally for the purpose of determining the number of bacteria present in a given specimen. It is obvious, of course, that the mere determination of the number of bacteria present in milk is no definite index as to whether or not it is safe for human consumption. A small number of typhoid, scarlet fever or diphtheria organisms would render milk of a low total bacterial count very dangerous, while, on the other hand, milk containing hordes of non-pathogenic bacteria might be consumed with no ill effects whatever. The value of the routine bacterial count lies in the fact that the number of bacteria present is an index to the efficacy of the methods of production and handling. A high bacterial count over a continued period ordinarily is indicative of unsatisfactory conditions at the dairy; careless and inefficient methods. Milk produced under such conditions is, obviously, more apt to become contaminated with organisms detrimental to the health of man than milk produced under ideal conditions.

In making routine bacterial counts of milk the standard methods recommended by the American Public Health Association are generally employed. The methods briefly described below are based on such standards. For a more detailed discussion of the methods the reader is referred to the pamphlet—"Standard Methods of Milk Analysis," published by the American Public Health Association.

PLATING METHOD

The plating method is the standard method commonly employed by most laboratories.

Collection of Samples. If a sample is to be taken from a can or other container all equipment used in procuring same should, obviously, be sterile. Before taking specimens from such containers the milk should be thoroughly mixed and 10 cc. or more drawn with a sterile tube or pipette, endeavoring to get a representative specimen of the entire column of milk (top to bottom). The sample is placed in a suitable, sterile bottle, properly stoppered, immediately iced and transmitted to the laboratory. If the specimen consists of one or more bottles of milk, put up by the dairy, these are simply well iced and sent to the laboratory with as little delay as possible.

Plating. The samples should be plated as soon after collection as possible. In carrying out the method the use of a standard beef-extract culture medium is specified. This consists of the following:

Agar	15 grams
Beef Extract (Liebig's or equivalent)	3 grams
Peptone	5 grams
Distilled water	1,000 cc.

The reaction of the medium must be between pH 6.2 and 7.0. Medium prepared in accordance with the above formula ordinarily possesses a reaction within the allowable range and thus needs no adjustment. Its reaction, however, must be determined to make sure that it is within such range.

The medium is tubed in 10 cc. amounts and sterilized by autoclaving at 15 pounds pressure for 20 minutes or by heating in flowing steam in the Arnold sterilizer for 20 minutes on each of three successive days.

In carrying out the plating three dilutions of the milk are made in sterile, distilled water—1:100, 1:1000 and 1:10,000. After thoroughly mixing, exactly 1 cc. of each dilution is placed in sterile Petri dishes, each of which is properly marked to indicate the particular dilution of milk. In the meantime a sufficient number of tubes of the beef-extract agar are placed in a boiling water bath and the medium melted and allowed to cool down to 42° to 45°C. A tube of such medium is poured into each plate containing the diluted milk and a mixture accomplished by carefully rotating the plate. After the agar has hardened the plates are inverted and incubated for 48 hours at 37.5°C. At the end of such period the colonies are counted.

Plates containing from 30 to 300 colonies should be counted and the bacterial content of the milk determined by multiplying by the particular dilution figure. Where more than one plate can be counted the final figures (count multiplied by dilution) for each plate should be added and averaged to give the bacterial contents of the particular specimen.

When it is known or suspected that a specimen of milk will run low in

bacteria, plating of the higher dilution can be omitted. On the other hand, where evidence is available that the count will be very high the two smaller dilutions can be dispensed with and several plates representing dilutions greater than 1:10,000 substituted.

In reporting bacterial counts of milk it has been common practice to indicate that a particular specimen was found to contain a definite number of bacteria per cubic centimeter. The American Public Health Association recommends that in place of stating definitely that a specimen contains a certain number of organisms per cubic centimeter, to indicate that the official plate count gives a bacterial count of a particular number of bacteria. Thus, where a specimen gives a count of 45,000 it is reported as follows: "Official plate count 45,000." Such expression means that according to the standard method of making counts, a bacterial content of a certain number per cubic centimeter of milk was obtained. It is recognized that the plate method only gives comparative figures and does not indicate definitely the exact number of bacteria in a given sample of milk. Because of such fact the above phrase is recommended.

BREED DIRECT MICROSCOPIC METHOD

Breed and Brew[1] have advocated a direct microscopic method of determining the bacterial content of milk which has been approved as a standard method by the American Public Health Association.

In this method a representative specimen of the milk is obtained and a minute amount of same drawn into a special capillary pipette which will discharge exactly 0.01 cc. of milk. Such pipettes can be contructed from glass tubing and carefully calibrated or they can be purchased from firms handling laboratory supplies.

In addition to the capillary pipettes, a special glass slide, with one or more ruled areas one centimeter square, is required to serve as a guide for spreading the 0.01 cc. of milk over the proper surface. Such ruled slide can be purchased from various supply houses.

In proceeding with the examination 0.01 cc. of the well-mixed milk sample is discharged from the special pipette on a clean slide and carefully spread over 1 square centimeter of the slide's surface. Such area is readily indicated by placing a plain slide over the special ruled slide which serves as a guide. Of course, if desired, a number of ruled slides can be kept on hand and the preparation made directly upon those. However, the single ruled slide used as a guide with plain slides will suffice.

[1] Breed and Brew, N. Y. Agric. Exp. Sta., Tech. Bull. No. 49, 1916.

After the milk has been thus spread the film is allowed to dry. It is then placed in xylol for one or two minutes to remove the fat. The xylol is drained off and the preparation allowed to dry, after which it is placed in methyl alcohol for two minutes. The preparation is then treated with Loeffler's methylene until it is overstained. Differentiation is then carefully accomplished with 95 per cent alcohol so that the background of the film appears a very pale blue and the bacteria a deeper blue.

Before counting the bacteria the size of the field obtained with the 1.9 mm. objective and the ocular used, must be ascertained for the particular microscope. This can be readily accomplished with a micrometer slide. Using a suitable ocular (usually 6.4×), manipulate the draw tube of the microscope until the area of the field is approximately 0.003 cm. Such field contains the dried, solid material of $\frac{1}{30,000}$ cc. of the milk specimen. For milk containing a fair number of bacteria thirty or more such fields are counted and the total bacteria in 1 cc. of milk computed. For example, the total bacterial content of thirty fields is found to be 64. The thirty fields represent $\frac{30}{30,000}$ or $\frac{1}{1000}$ cc. of the milk specimen. Thus $64 \times 1000 =$ 64,000 organisms per cubic centimeter of milk.

When milk of low bacterial content is examined by this method a special ocular micrometer is used. The microscope is so adjusted that the field in the ocular circle is 0.146 mm., such field representing $\frac{1}{600,000}$ cc. of the milk specimen. The bacteria in 60 of these fields are counted and the total for the sample computed.

The Breed method for estimating the bacterial content of milk results in counts three or four times as great as those obtained by the standard plating method. This difference is due to the fact that some of the organisms observed microscopically are dead, or may not grow on the culture medium employed, and, further, clumps of bacteria consisting of a number of organisms will produce but one colony in plate cultures.

CERTIFIED MILK

Certified milk is produced under the supervision of a Medical Milk Commission, the certificate of such commission indicating that the producer is complying with various specified requirements, sufficiently stringent to reasonably insure a safe, wholesome, raw milk. The exact requirements differ somewhat with different commissions. In the main, they cover, in a strict manner, the health and care of the animals, health of employees, equipment, sanitation, methods, storage, delivery, and chemical constituents and bacterial content of the milk.

In the bacteriological examination of certified milk the official plate count must not exceed 10,000.

PASTEURIZATION OF MILK

Pasteurization is a process by which milk is heated to a temperature sufficiently high to destroy pathogenic bacteria. Further, by destroying a large percentage of the bacteria present, pasteurization improves the keeping quality of milk. The process has as a main object the destruction of Mycobacterium tuberculosis which is very frequently present in milk from dairies where tuberculous cows are maintained. The procedure also serves to destroy the etiological agents of typhoid, diphtheria, scarlet fever, etc., should they find their way into the milk. The temperatures employed in pasteurization, however, are not such as will destroy all bacteria. Various resistant saprophytes and a few pathogens (the anthrax organism for example) are not affected. Fortunately, pathogenic forms which would survive pasteurization are not very frequently found in milk.

Two methods of pasteurization have been advocated. One is known as the *flash* method, the other the *holder* process. At the present time the latter process is the one generally employed. The flash method consists in quickly heating the milk to a temperature of 185°F. and immediately cooling it. In the holder process the temperature of the milk is raised to 145° to 147°F. and maintained at such for 30 minutes and then rapidly cooled.

BACTERIOLOGICAL EXAMINATION OF WATER

Bacteriological examination of water is an important procedure in determining its potability. Formerly, considerable weight was given to the total number of bacteria present in water. During recent years, however, such quantitative determinations have been shown to be more or less unreliable and consequently less weight is now attached to them although as a routine procedure bacterial counts are made.

It is recognized that evidence of fecal contamination of a water supply is a better index of potability than the number of bacteria in a given specimen. As the presence of organisms of the Escherichia (colon) group in appreciable numbers constitutes rather definite evidence of fecal contamination, present methods of examining water have as a principal object, the detection of such bacteria. In a rough way the standard test for organisms of the colon group is quantitative. Thus, if such organisms are present in considerable numbers, as would be the case where gross fecal contamination of the water has occurred, the same is indicated by gas formation in fermentation tubes inoculated with small amounts of the water and in several or all of the tubes containing large portions.

In conducting bacteriological examinations of water the standard methods

of the American Public Health Association should be followed. For full details of same reference is made to the association's "Standard Methods for the Examination of Water and Sewage."[2]

Determination of Bacterial Content of Water

In examinations to determine the number of bacteria present in water the plating method is employed.

A representative specimen of the water should be obtained in a sterile bottle and the plating accomplished with the least possible delay. The American Public Health Association's methods specify that the sample should be plated within six hours after procurement, in the case of impure waters, and within twelve hours when relatively pure waters are examined. In the meantime the water should be kept at a temperature between 6° and 10°C.

The dilutions of the water for plating will depend somewhat on the particular specimen. However, it is desirable to make the dilutions so that several plates will develop from 30 to 300 colonies per plate. Ordinarily, plates made from the straight water, a 10 per cent dilution, and 1 per cent dilution, respectively, are satisfactory.

In carrying out the plating, the straight water and dilutions are placed in 1 cc. amounts in sterile Petri dishes. Then 10 cc. of melted gelatin or agar, cooled down to 40°C., are poured into each dish and a thorough mixture of water and medium accomplished by rotating the dish. After solidification the gelatin plates are incubated for 48 hours at approximately 20°C. Agar plates can likewise be incubated for 48 hours at 20°C., or they may be incubated at 37°C. for 24 hours. After such incubation period the plates are examined and those showing from 30 to 300 colonies used for making the count. Obviously, if the plate which received undiluted water contains less than 30 colonies the 30 to 300 rule would be disregarded. Where several plates can be counted the counts are added and averaged.

While, as previously indicated, it is impossible to accurately determine the potability of a specimen of water from a bacterial count, it is more or less generally accepted that water producing not more than 100 colonies per cubic centimeter is potable; approximately 500 colonies suspicious; and over a thousand colonies not potable.

The media employed are made according to the standard methods outlined by the American Public Health Association. In the preparation of

[2] Published by American Public Health Association, 370 Seventh Avenue, New York, N. Y.

broth, beef-extract in the amount of 3 grams and peptone 5 grams are added to 1000 cc. of distilled water. The reaction is adjusted so that the final pH will be between 6.2 and 7.0. Sterilization is accomplished by autoclaving for 15 minutes at 15 pounds pressure.

The agar is prepared by using the same amounts of ingredients employed for broth plus 15 grams of commercial agar. The final reaction should be between pH 6.2 and 7.0. Sterilization is accomplished by 15 minutes exposure to 15 pounds steam pressure.

Gelatin medium is prepared by using the broth ingredients in the amounts specified and 120 grams of gelatin. The final pH should be between 6.2 and 7.0. Sterilize by autoclaving at 15 pounds pressure for 15 minutes.

Tests for Organisms of the Escherichia (Colon) Group

The basis for the tests used in detecting colon organisms lies in the fact that Gram-negative, non-sporulating, rods, which split lactose with the formation of gas and grow aerobically on solid-medium, when present in water, can be considered as belonging to the colon group.

In conducting routine tests the procedure commonly employed is to inoculate one or preferably two sets of 7 fermentation tubes of lactose broth. Five tubes are inoculated with 10 cc. portions of the water under examination and the other two with 1.00 cc. and 0.1 cc. respectively. Each tube should contain at least twice as much medium as the amount of water inoculated into same. The Dunham type fermentation tube with inverted phial is recommended.

After inoculation the tubes are placed in the incubator at 37°C. and examined after 24 and 48 hours. Formation, within 24 hours, of gas occupying more than 10 per cent of the space in the inverted phial, constitutes a *positive presumptive test* for Escherichia organisms. If within 24 hours no gas is formed or if less than 10 per cent is produced the incubation is continued to 48 hours and the presence of any amount of gas at the end of such period constitutes a *doubtful test* and must, invariably, be confirmed. If after 48 hours incubation no gas is formed the test is considered *negative.*

To confirm a fermentation reaction, select the tube showing gas formation from the smallest amount of water and inoculate one or more plates of Endo medium or eosin-methylene-blue agar with a loopful of the culture. Such plates should be inoculated at the 24-hour reading of the fermentation tubes if gas is then present, rather than after 48 hours. The plates are incubated for 18 to 24 hours at 37°C. If typical colonies develop the test can be considered confirmed as positive. Then, to complete the test, at least two typical colonies are fished from the plate and transferred to a

lactose broth fermentation tube and an agar slant. Gas formation in the lactose-broth medium and the demonstration of the growth on the agar slant to be made up of Gram-negative, non-sporulating bacilli, constitutes a *completed positive test.*

Where no typical colonies develop on the Endo or eosin-methylene-blue plates within 24 hours the incubation is continued for another 24 hours and then at least two colonies which are considered most likely to belong to the Escherichia group are selected and inoculated into lactose-broth and on agar. The development of gas in the fermentation tube and the demonstration of Gram-negative, non-sporulating bacilli in the agar culture constitute a positive completed test. The absence of gas and the demonstration of an organism of a type other than that indicated is considered negative.

The lactose-broth employed in the fermentation tests is prepared by adding 0.5 per cent lactose to the standard broth, the preparation of which has already been given. The Endo medium and eosin-methylene-blue agar are prepared in accordance with standard methods (see page 58).

With potable water not more than one out of the 5 tubes containing 10 cc. portions of water should be positive for Escherichia organisms. Badly contaminated water will be indicated by positive results with the cultures containing the smaller portions of water.

INDEX

Abbe, 29
Abbott's method of staining spores, 97
Abortion, infectious, bovine, 175
  agglutination test for, 446
  complement-fixation test for, 438
  organism of, 175
  preparation of vaccine for, 473
Abortion, infectious, equine, 163
  agglutination test for, 446
  complement-fixation test for, 438
  immunization against, 165
  organism of, 163
Accredited Herds, tuberculosis free, 267
Acetyl-methyl-carbinol, test for, 54
Achorion canis, 315
  gallinarum, 314
  genus, organisms of, 313
  quinckeanum, 315
  schoenleini, 314
Achromatiaceae, general description of, 17
Acid-fast bacteria, 263
  methods of staining, 89
  saprophytic, 283
Acids produced by bacteria, 15
Actinobacillus, 242
  genus, specifications of Com. Soc. Amer. Bact., 242
  lignieresi, 242
    cultural characteristics of, 243
    morphology and staining of, 243
    pathogenicity of, 243
    resistance of, 243
Actinomycetaceae, general description of, 17
Actinomycetales, general description of, 16
Actinomyces, 244
  actinoides, 249
    cultural characteristics of, 250
    differentiation from Actinobacillus lignieresi, 249
    morphology and staining of, 250
    pathogenicity of, 250
  bovis, 244
    cultural characteristics of, 246
    morphology and staining of, 245
    pathogenicity of, 249
    resistance of, 248
  farcinicus, 251
    cultural characteristics of, 252
    morphology and staining of, 251
    pathogenicity of, 252
    resistance of, 252
  genus, specifications of Com. Soc. Amer. Bact., 244
  necrophorus, 253
    cultural characteristics of, 255
    morphology and staining of, 254
    pathogenicity of, 256
    resistance of, 256
  nocardii, 251
Actinomycosis, organism of, 244
Adil-Bey, 412
Aerobacter, 156
  aerogenes, 156
  genus, specifications of Com. Soc. Amer. Bact., 156
Aerobes, cultivation of, 71
  obligatory, defined, 13
  strict, defined, 13
African horse sickness, virus of, 414
Agar, blood, 57
  chocolate, 57
  dextrose-serum, 61
  glucose, 57
  glycerin, 57
  hemoglobin, 60
  hormone, 62
  lactose-litmus, 58
  lead acetate, 62
  maltose, 57
  nutrient, 56
  one-tenth per cent (Hitchens), 63
  serum, 57

Agar, serum-glucose-cystine (Francis), 64
  starch (Vedder), 60
Agglomeration, in protozoa, 334
  in trypanosomes, 334, 344
Agglutination test, 442
  for bovine infectious abortion, 446
  for equine infectious abortion, 446
  for glanders, 444
  for Malta fever, 446
  for Salmonella pullorum infection, 447
  macroscopic, described, 443
  microscopic, described, 443
Agglutinin absorption tests, 447
Agglutinins, 115
  production of, Ehrlich's theory, 115
  theoretical structure of, 115
Agglutinogen, defined, 115
Aggressin, 105, 473
  "artificial," preparation of, 475
  blackleg, preparation of, 474
  hemorrhagic septicemia, preparation of, 475
  in anthrax immunization, 211
  in infection, 105
  preparation of, for immunization purposes, 473
  symptomatic anthrax, preparation of, 474
Alcaligines, 175
  abortus (Bang), 175
    carriers of, 176
    cultural characteristics of, 177
    differentiation of, from A. melitensis, 179
    immunization, 180
    mode of infection with, 177
    morphology and staining of, 177
    pathogenicity for males, 176
    pathogenicity for man, 175
    resistance of, 179
    serological reactions, 180
  bronchisepticus, 183
    cultural characteristics of, 184
    immunization against, 185
    morphology and staining of, 184
    resistance of, 185
  genus, specifications of Com. Soc. Amer. Bact., 175
  melitensis, 180
    cultural characteristics of, 182
    differentiation from A. abortus, 179
    morphology and staining of, 182
    resistance of, 183
    serological reactions, 183
Alexin, as related to immunity, 111
Alkali-pyrogallic acid method of cultivating anaerobes, 73
Allergy, defined and discussed, 119
Amboceptor, 118
  bacteriolytic, defined, 118
    in complement-fixation test, 422
  hemolytic, defined, 118
    in complement-fixation test, 422
    preparation and titration of, 425
Amitosis, in protozoa, fallacies, 330
Ammonio-ferric-alum, 358
"Amoeba meleagridis," 361
  flagellated stage in life cycle, 325
  identification of, 334
  of frogs, 341
  of lower animals, 341, 361
  of man, 341
  of monkeys, 341
  of rodents, 341
  parasitic, 339, 341
  see Sarcodina
Amoebae, commensal types, 341
Anaerobes, alkali-pyrogallic acid, method of cultivating, 73
  Buchner's method of cultivating, 74
  cultivation in Brown's jar, 76
  cultivation of, 71
  facultative, defined, 13
  growth in symbiosis with other bacteria, 81
  inoculating media with, 70
  Krumwiede & Pratt's method of cultivating, 76
  McIntosh & Fildes' method of cultivating, 76
  obligatory, defined, 13
  oxygen displacement method of cultivating, 72
  oxygen exclusion method of cultivating, 72

Anaerobes, oxygen exhaustion method of cultivating, 72
strict, defined, 13
tissue enrichment method of cultivating, 75
Wright's method cultivating, 75
Zinsser's method of cultivating, 75
Anal pore, in protozoa, 328
Anaphylaxis, defined and discussed, 119
nature of reaction, 121
seat of reaction, 121
Wells' criteria for determining, 123
Anaplasma, 383
centrale, 385
differentiation of species, 350
genus, varieties of, 383
marginale, 384
morphology of 384
transmission of, 385
(variety centrale), 385
Anaplasmosis, bovine, organism of, 384
Anderson, 120
Anemia, infectious, equine, virus of, 408
Aniline-gentian-violet, 87
Anthrax, 203
Ascoli's test for, 448
bacillus, 203
complement-fixation test for, 439
precipitation test for, 448
serum, preparation of, 476
serum therapy and prophylaxis, 208
spore vaccine, preparation of, 472
susceptibility of man to, 203
vaccination, 208
aggressins in, 211
Chauveau's method, 211
Pasteur's method, 209
serum and spore vaccine method, 211
simultaneous method, 210, 472
Sobernheim's method, 210
symptomatic, 213
immunization against, 217
organism of, 213
preparation of aggressin for, 474
preparation of culture filtrate for immunization against, 475
preparation of vaccines for, 472
Anti-aggressins, in immunity, 106
Antigens, defined, 112
Antitoxin, 476
botulism, preparation of, 481
production of, Ehrlich's theory, 113
tetanus, preparation and standardization of, 479
tetanus, unit defined, 480
Aphthous fever, virus of, 410
Arloing, 213, 217, 218
Armanni, 195
Arnold sterilizer, 33
Arthropoda, intermediary hosts of trypanosomes, 344
in transmission of protozoa, 337
in transmission of trypanosomes, 343
mechanical transmitters of trypanosomes, 344
Arthus, 120
phenomenon of, 120
Artifacts, in staining protozoa, 355
Ascoli's test for anthrax, 448
Ascomycetes, 309
genus Achorion, 313
genus Aspergillus, 315
genus Microsporum, 312
genus Trichophyton, 309
Ascospores, production of, by fungi, 302
Asexual phase, of coccidia, 347
Aspergillosis, organisms causing, 315
Aspergillus, 315
fumigatus, 316
nigrescens, 316
Asterococcus mycoides, 404
Atmospheric requirements of bacteria, 12
Aufrecht, 264
Autoclave, 33
temperatures obtained with, 36
Avian diphtheria, virus of, 416
Avian tuberculosis, 279
Axial filament, in Sarcodina, 339
Axostyle, in Trichomonas, 333
Ayers, 136, 137

Babes, 7, 289, 374, 381
Babesia, 349, 374
bigemina, 374
carriers of, 377
immunization against, 377

Babesia, bigemina, morphology of, 375
resistance of, 375
transmission of, 377
budding in, 350
canis, 380
cultivation of, 381
morphology of, 380
transmission of, 381
"chromidia" in, 349
cultivation of, 349
development in tick, 350
equi, 377
morphology, 378
transmission, 378
gamete formation, 350
germinal transmission of, 338
infection of corpuscles, 349
infection of vertebrate host, 350
intracorpuscular stage, 349
life cycle, 349
mutans, 379
morphology of, 379
transmission of, 379
oökinete stage, 350
ovis, 381
morphology of, 381
transmission of, 381
reproduction in, 350
structure of nucleus, 349
zygote formation, 350
Bacillaceae, general description of, 17
Bacillary dysentery, chronic, bovine, organism of, 280
Bacilli, general description of, 8
Bacillus abortivo-equinus, 163
abortus (Bang), 175
abortus–equi, 163
actinoides, 249
aerogenes, 156
aerogenes capsulatus, 237
aertrycke, 160
anthracis, 203
cultural characteristics, 207
immunization against, 208
morphology and staining, 206
resistance of, 208
serological reaction, 208
avisepticus, 194
bipolaris septicus, 186
botulinus, 229
bovisepticus, 188
bronchicanis, 183
bronchisepticus, 183
bubalsepticus, 195
butyricum, 283
chauvei, 213
cholera suis, 161
coli communior, 155
coli communis, 153
diphtheriae vitulorum, 253
enteritidis, 160
equisepticus, 191
erysipelatus suis, 258
filiformis, 253
gallinarum, 168
gangraenae emphysematosae, 213
genus, specifications of Com. Soc. Amer. Bact., 201
leprae, 284
lignieresi, differentiation from Actinomyces actinoides, 249
mallei, 289
murisepticus, 262
necrophorus, 253
oedematis-maligni, 219
of blackleg, 213
of Bradsot, 219
of enteritis paratuberculosis bovis, 280
of gastromycosis ovis, 219
of mouse typhoid, 162
of pseudotuberculosis, 286
paratuberculosis, 280
paratyphosus "A", 170
paratyphosus "B", 157
perfringens, 237
pestis, 195
pestis caviae, 160
phegmonis emphysematosae, 237
phlei, 283
psittacosis, 163
pullorum, 167
pyocyaneus, 149
rhusiopathiae suis, 258
smegmatis, 283
subtilis, 201
cultural characteristics, 201

Bacillus subtilis, morphology and staining, 201
resistance of, 202
suipestifer, 161
suisepticus, 192
tetani, 223
tuberculosis, 263
tuberculosis avium, 279
tuberculosis (bovis), 266
tularense, 197
typhi-murium, 162
typhosus, 171
welchii, 237
Bacteria, 8
acid-fast, 263
methods of staining, 89
saprophytes, 283
acids produced by, 15
aerobic, cultivation of, 71
anaerobic, cultivation of, 71
artificial propagation of, 65
atmospheric requirements, 12
capsules of, 9
chemical composition of, 10
classification chart, 18
classification of, 15
colon, 152
cultivation of, 71
cultivation of, in atmosphere rich in CO_2, 81
definition of, 8
degenerative forms, 9
detection of gas produced by, 81
determination of motility of, 83
enzymes produced by, 15
facultative pathogenic, defined, 101
families of, 16
family names, ending of, 17
gases produced by, 15
genus, Actinobacillus, organisms of, 242
Actinomyces, organisms of, 244
Aerobacter, organisms of, 156
Alcaligines, organisms of, 175
Bacillus, organisms of, 201
Borrelia, organisms of, 295
Clostridium organisms of, 213
Corynebacterium, organisms of, 285
Eberthella, organisms of, 171
Erysipelothrix, organisms of, 258
Escherichia, organisms of, 152
Mycobacterium, organisms of, 263
Pasteurella, organisms of, 186
Pfeifferella, organisms of, 289
pseudomonas, organisms of, 149
Salmonella, organisms of, 157
Staphylococcus, organisms of, 141
Streptococcus, organisms of, 127
Vibrio, organisms of, 146
Gram-negative organisms defined, 85
Gram-positive organisms defined, 85
influences detrimental to, 14
involution forms, 9
media reaction requirements, 13
metachromatic granules of, 10
methods for destruction of, 31
microscopic study of, 83
morphology of, 8
nutritional and temperature requirements, 11
orders of, 16
parasitic, defined, 101
non-pathogenic, defined, 101
pathogenic, defined, 101
physiology of, 11
pigments of, 15
pleomorphism of, 9
products of growth of, 15
reproduction of, 11
saprophytes, defined, 101
Society of American Bacteriologists' classification of, 16
spores of, 10
stain preparations, 84
structure of, 10
sub-families of, 22
temperatures in cultivation of, 71
toxins of, 15
transmutation of, 9
tribes of, 22
Bacteriaceae, general description of, 17
Bacterins, 463
definition of, 463
standardization, methods for, 465
technic for preparation of, 464
Bacteriological methods, 23
Bacteriology, history of, 3

Bacteriolysins, 112, 116
Ehrlich's theory of production, 116
Bacteriolysis, defined, 117
Bacteriolytic amboceptor, in complement-fixation test, 422
Bacteriolytic system, in complement-fixation test, defined, 423
Bacteriophage, 14
Bacteriophagy, 14
Bacterium aborti-equi, 163
abortus (Bang), 175
abortus-equi, 163
acidi-lactici, 156
anatum, 165
bronchicanis, 183
bronchisepticus, 183
gallinarum, 168
jeffersonii, 172
lactic aerogenes, 156
mallei, 289
melitensis, 180
pfaffi, 173
psittacosis, 163
pullorum, 167
pyocyaneum, 149
rettgeri, 174
sanguinarium, 168
tuberculosis avium, 279
tuberculosis bovis, 266
tularense, 197
Baeslack, 298
Bail, 105, 106, 211, 473
Balantidiosis of hogs, 390
Balantidium, 353, 389
coli, 353, 354, 390
encystation, 354
life cycle in pig, 354
morphology of, 354
tissue parasite, 327
conjugation in, 355
contractile vacuole in, 328
dividing forms in feces, 355
encystation in pig, 355
in caecum of pig, 355
incidence of infection in pigs, 355
in ileum of pig, 355
in invertebrate hosts, 353
in man, 354
in vertebrate hosts, 353
life cycle, 355
pathogenicity of, 354
site of infection in pigs, 355
species in the pig, 353
structure of nuclei, 331, 332
minutum, 354
suis, 390
morphology of, 354
Bang, 175, 281
Banzhaf, 480
Basal granules, in Protozoa, 328, 342
relation of, to flagellum, 333
Bass, 338, 349
Baumgarten, 264
Beach, 282, 417
Beggiatocaceae, general description of, 17
Behring, 7, 111
Belfanti, 116
Bengston, 230, 236
Bergey, v, 16
Bertarelli, 394
Biliary fever, canine, organism of, 380
Binucleata, 332
Biological products, veterinary, preparation of, 461
Blackhead, in turkeys, 344
"Amoeba meleagridis" in, 361
organism of, 372
Blackleg, 213
aggressin, preparation of, 474
culture filtrate, preparation of, 475
organism of, 213
preparation of vaccines for, 472
Black water, bovine, organism of, 374
Blastomyces, 305
farciminosus, 305
cultural characteristics, 307
morphology and staining, 306
pathogenicity for man, 306
pathogenicity of, 308
resistance of, 308
Blastomycetes, 301
Blastomycosis, equine, organism of, 305
Blepharoplast, 333, 342
Blood broth, 56
Blood, cat, cellular constituents of, 460
cell counts, diluting fluids for, 455

Blood, cellular constituents of normal animals, 460
clinical examination of 453
counts, technic of, 453
cow, cellular constituents of, 460
differential white cell counts, 459
dog, cellular constituents of, 460
enumeration of red cells, technic of, 456
hemoglobin, methods for estimating, 458
horse, cellular constituents of, 460
protozoa, fixation and staining of, 355
stain, Giemsa's, 92
stain, Wright's, 91
swine, cellular constituents of, 460
white cell counting, technic of, 457
Boeck, 339
Bollinger, 6, 188, 213, 244
Bolton, 406
Bordeaux red, for protozoa, 358
Bordet, 106, 111, 112, 116, 449
Borrel, 404
Borrelia, 295
gallinarum, 295
biological therapy and prophylaxis,, 297
cultivation of, 296
morphology and staining, 296
pathogenicity of, 296
hyos, 298
theileri, 297
Bosc, 394
Botulism, antitoxin, preparation of, 481
organism of, 229
serum therapy and prophylaxis, 235
Bouin's fluid, 357
Bovine coccidiosis, organism of, 387
farcy, organism of, 251
malaria, organism of, 374
piroplasmosis, transmission, 351
Boyle, 5
Boynton, 413
Bradsot, organism of, 219
Brain medium, Hibler's, 63
Brauell, 5, 203
Braxy, organism of, 219
Breed, v, 489
method for determining bacterial content of milk, 489
Brew, 489
Broden, 370
Broth, beef extract, 51
beef infusion, 50
calcium carbonate, 61
glycerin, 51
hemoglobin, 61
Martin's peptone, 52
sugar-free, 51
Brown, 76, 84, 128, 129
anaerobe jar, method of making, 76
modification of McIntosh and Fildes' method of cultivating anaerobes, 76
Brownian movement, 83
Bruce, 180, 365
Brucella abortus (Bang), 175
melitensis, 180
Brumpt, 350, 359
Bubonic plague, organism of, 195
Buchanan, 16
Buchner, 106, 111
method of cultivating anaerobes, 74
Buck, 81, 176, 179, 437
Budding, in Infusoria, 352
in protozoa, 325
process of, by Blastomycetes, 301
Buffalo, hemorrhagic septicemia of, organism of, 195
Buffers, described, 46
Butter bacillus, 283

Cagniard-Latour, 5
Calf diphtheria, organism of, 253
Calkins, 324, 325, 326, 395
Callender, vi
Canine distemper, Alcaligines bronchisepticus in, 183
virus of, 411
Capsules, of bacteria, 9
staining of, 93
Carbohydrases, production of, by bacteria, 15
Carbohydrate media, 52
Carbone, 117
Carceag, of sheep, organism of, 381
Carle, 223
Carnwath, 416
Carpano, 297

Carre, 409, 411
Carriers, mechanical, of protozoa, 337
Caseous-lymphadenitis of sheep, organism of, 286
Cats, coccidia of, 346
"Cattle plague," virus of, 412
Cell inclusions, in protozoa, 329
Centanni, 415
Centriole, 331
Centrosome, in protozoa, 328, 331, 342
  relation to blepharoplast, 333
Cerebro-spinal meningitis, equine, C. botulinum in, 229
Certified milk, 490
Chabert, 213
Chamberland, 209, 394
Chapin, 198
Charbon, organism of, 203
  symptomatic, organism of, 213
Chauveau, 211
Chemotaxis, positive, defined, 108
Chester, 16
Chilomastix, 328, 342
Chlamydobacteriaceae, general description of, 17
Chlamydobacteriales, general description of, 16
Chlamydospores, 304
Chlamydozoa, in avian pox and diphtheria, 417
Cholera, fowl, organism of, 194
  hog, Pasteurella suiseptica in, 192
  hog, Salmonella suipestifer in, 161
Chromatin, peripheral in protozoan nucleus, 331
  staining reactions in protozoan nucleus, 329
Chromatoidal bodies, in Entamoeba coli, 341
  in parasitic amoebae, 329
"Chromidia," in Babesia, 349
Chromosome counts, in protozoa, 333
Chromosomes, in protozoa, 333
Cilia, in protozoa, 328
  distinction from flagella, 352
Ciliata, 352, 389
Cirri, 328, 352
Classification,
  bacteria, 15
  chart illustrating same, 18
  Society of American Bacteriologists' system, 16
  of fungi, 302
  of protozoa, characters of main groups, 326, 360
  Schaudinn's basis for, 325
  of streptococci, 127
Clinical blood examinations, 453
Clostridium, 213
  botulinum, 229
    cultural characteristics of, 232
    identification of, by complement-fixation test, 439
    identification of, in canned food-stuffs, 235, 441
    morphology and staining of, 232
    pathogenicity of, 235
    production of toxin by, 233
    resistance of, 234
    serological reactions, 235
    serum therapy and prophylaxis, 235
    toxin, identification of, in canned food-stuffs, 235
    types of, 230
  chauvei, 213
    cultural characteristics of, 215
    immunization against, 217
    morphology and staining of, 214
    pathogenicity of, 217
    resistance of, 216
    serological reactions, 217
    toxin of, 216
  genus, specifications of Com. Soc. Amer. Bact., 213
  hemolyticus bovis, 239
    cultural characteristics of, 240
    morphology and staining of, 240
    pathogenicity of, 241
    production of toxin by, 241
  luciliae, 236
    cultural characteristics of, 236
    morphology and staining of, 236
    pathogenicity of, 237
    resistance of, 237
    toxin of, 236
  oedematis-maligni, 219
    cultural characteristics of, 222

Clostridium oedematis-maligni, morphology and staining of, 221
pathogenicity of, 223
production of toxin by, 223
resistance of, 223
of bradsot, 219
cultural characteristics of, 219
morphology and staining of, 219
pathogenicity of, 219
tetani, 223
carriers of, 224
cultural characteristics of, 225
immunization against, 229
in soil, 224
morphology and staining of, 225
production of toxin by, 227
resistance of, 228
tetanolysin produced by, 228
toxin, resistance of, 227
types of, 229
welchii, 237
cultural characteristics of, 238
morphology and staining, 237
pathogenicity of, 239
production of toxin by, 239
resistance of, 239
types of, 239
Coagulase, production of, by bacteria, 15
Coccaceae, general description of, 17
Cocci, division of, 11
general description of, 8
Coccidia, 346, 385
cysts, in feces of uninfected animal, 337
formation of, 348
resistance of, 347
identification of, 334
infection with, 347
lethal species, 346
life cycle, 327, 347, 349
of cats, 346
of centipede, 346
of dogs, 346
pathogenicity of, 346
submucous tissue in, 346
syngamy in, 347
transmission of, 347
Coccidiosis, immunity in, 347
in dogs and cats, Isospora type, 388
life cycle of organism causing, 385
of birds, organism of, 386
of cattle, organism of, 387
of Philippine lizards, 337
of rabbits, 346, 386
organisms of, 385
pathology of, 346
reinfection in, 347
transmission by lizards, 338
Coccidium, 348, 385
avium, 386
cuniculi, 386
oviforme, 386
schubergi, 346
tenellum, 386
zürni, 387
Cohn, 5, 16, 81
Cole, 62, 386
Colitis, balantidal, 354
Colon organisms, 152
Columella, of fungi, 304
Commensalism, defined, 13
with protozoa, 336
Commensals, defined, 14
Complement, its part in immunity, 112
Complement-fixation test, 422
bacteriolytic amboceptor in, 422
bacteriolytic system defined, 423
for anthrax, 439
for bovine infectious abortion, 438
for equine infectious abortion, 438
for glanders, 424
for Malta fever, 439
for trypanosomiasis, 436
hemolytic amboceptor in, 422
hemolytic system defined, 423
identification of C. botulinum and its toxin by, 441
of cultures, bacterins, etc. by, 442
preparation and titration of complement, 428
Complement, preparation and titration of, 428
Condenser, microscope, description of, 28
Conglutination test, for dourine, 449
for glanders, 449
technic of, 449
Congo red, for protozoa, 358

Conidia, of fungi, 304
Conjugation, in protozoa, 333
Contagious pleuro-pneumonia, bovine, 404
  immunization against, 405
  virus of, 404
Contractile vacuole, in protozoa, 328
Copulation, in protozoa, 333, 334
Cornevin, 213, 217, 218
Corpuscles, Rainey's, 351
Corynebacterium, 285
  diphtheriae, 285
    carriers of, among lower animals, 285
  genus, specifications of Com. Soc. Amer. Bact., 285
  ovis, 285
  pseudotuberculosis, 286
    cultural characteristics of, 287
    morphology and staining of, 286
    pathogenicity of, 288
    resistance of, 288
Cow-pox, virus of, 417
Creech, 176, 258
Crithida, 342
  relation to Trypanosoma, 342
Cryptococcus farciminosus, 305
Cultivation of aerobes, 71
  of anaerobes, 71
  of bacteria, 71
  of bacteria in atmosphere rich in CO_2, 81
Culture filtrates, for immunization purposes, preparation of, 475
Culture media, 44
  agar, blood, 57
    chocolate, 57
    dextrose-serum, 61
    glucose, 57
    glycerin, 57
    hemoglobin, 60
    lactose-litmus, 58
    lead acetate, 62
    maltose, 57
    nutrient, 56
    one-tenth per cent (Hitchens), 63
    serum, 57
    serum-glucose-cystine (Francis), 64
    starch (Vedder), 60
  beef extract broth, 51
    infusion broth, 50
  blood broth, 56
  brain medium (Hibler), 63
  broth, calcium carbonate, 61
    hemoglobin, 61
  carbohydrate, 52
  dextrose-serum broth, 56
  Dunham's peptone solution, 53
  egg (Dorset), 59
    glycerin, 59
  Endo's, 58
  enrichments for, 13
  eosin-methylene-blue, 58
  Francis' serum-glucose-cystine agar, 64
  gelatin, nutrient, 57
  glycerin broth, 51
    potato, 60
  Hibler's brain, 63
  Hiss serum-water, 55
  hormone or vitamine agar, 62
  inoculation of, 65
    with anaerobes, 70
  lactose-bile, 55
  litmus milk, 54
  liver broth, 54
  Loeffler's serum, 60
  Lubebau's glycerin-egg, 59
  Martin's peptone broth, 52
  meat mash for anaerobes, 63
  method of determining hydrogen ion concentration of, 45
  milk whey, 54
  Nicolle, Novy, MacNeal (N.N.N.), 63
  Petroff's, 59
  phenolphthalein titration method of adjusting reaccion, 49
  pork broth, 54
  potato, 59
    broth, 54
  reaction, methods for determining, 44
    requirements for bacteria, 13
  Russell's double sugar, 61
  Sabouraud's for fungi, 57
  serum broth, 56
  spreaders for inoculating, 67
  sterilization, 33, 36
    by filtration, 36
  sugar-free broth, 51
  Teague's 58

Culture media, veal broth, 54
Vedder's starch agar, 60
Cultures, plate, 68
pure, methods of obtaining, 68
stab, 70
Cumming, 403, 470
method of preparing rabies vaccine, 470
Cyanophaceae, relation to spirochaetes, 342
Cycloposthium, 353
Cyst, formation of, in coccidia, 348
of Entamoeba coli, 341
of Isospora bigemina, 347
of protozoa, 337
Cytolytic substances, production of, by bacteria, 15
Cytoplasm, in protozoa, 326
Cytoplasmic inclusions, in protozoa, 329
Cytopyge, defined, 328
Cytostome, defined, 328

Dahmen, 410
Dale, 122
D'Amato, 394
Dare's method of estimating hemoglobin, 458
Dark field examinations, 29
Darling, 368
Davaine, 5, 203
"Deer-fly fever," organism of, 197
Delafield, hematoxylin, 357
Delafond, 203
Dermatitis, equine, due to Fusarium equinum, 319
gangrenous, equine, organism of, 253
de Schweinitz, 406
Dextrose-serum broth, 56
d'Herelle, 14
"Diamond skin disease," swine, organism of, 258
Diarrhea, white, of chicks, organism of, 167
Dick, 131, 132
Dieckerhoff, 407
Digestive vacuoles, in protozoa, 329
Dimorphic nuclei, of ciliates, 330
Diphtheria, avian, virus of, 461
calf, organism of, 253
"Diplococcus pneumoniae," see Streptococcus equi, 133
Diplodinium, 353
ecandatum, 355
Diplospora, 348
Discomyces bovis, 244
farcinicus, 251
Disinfectants, 40
Distemper, canine, Alcaligines bronchisepticus in, 183
virus of, 411
Division, in Infusoria, 334, 352
in Mastigophora, 334
Dobell, 324, 341
Doflein, 359
Dog, amoebic dysentery in, 341
Doi, 403, 470
Donovan, 371
Dorset, 406, 478
egg medium, 59
Douglas, 108
Dourine, complement-fixation test for, 436
conglutination test for, 449
trypanosome of, 366
Drug idiosyncrasies, 123
Dubovsky, 441
Dunham's peptone solution, 53
Durham, 112
tubes, use of, 81
Dusch, 4
Dysentery, amoebic, cultivation of parasite, 339
balantidial, 354
in lower animals, 341

East African Coast fever, organism of, 382
Eberthella, 171
genus, specifications of Com. Soc. Amer. Bact., 171
jeffersonii, 172
cultural characteristics of, 172
morphology and staining of, 172
pathogenicity of, 172
resistance of, 173
pfaffi, 173
cultural characteristics of, 173
morphology and staining of, 173
rettgeri, 174
cultural characteristics of, 174
morphology and staining of, 174

Eberthella rettgeri typhi, 171
Ectoplasm, in Sporozoa, 328
of protozoa, structure and modifications of, 327
Ectothrix types of Trichophyton species, 310
Egg medium, Dorset's, 59
glycerin, 59
Ehrlich, 6, 106, 112, 113, 114, 115, 116
side chain theory of immunity, 112
test for indol, 53
Eichhorn, 211, 361, 403, 437
Eimeria, 346
avium, 386
bovis, 387
distinction from Isospora, 348, 388
genus, organisms of, 385
life cycle, 348-349
schubergi, 346
see also Coccidia, Coccidium
spores of, 347
stiedae, 386
Elmassian, 368
Emmerich, 262
Enchylema, 331
Encystation, in protozoa, 325, 337
Endo-ectothrix types of Trichophyton species, 310
Endogenous cycle in coccidia, 346
Endogenous infection, defined, 102
Endomyces albicans, 317
Endoplasm, modifications in parasitic protozoa, 329
of protozoa, general characters of, 326, 328, 329
Endo's medium, 58
Endothrix types of Trichophyton species, 310
Endotoxins, in infection, 105
defined, 15
Englenoid movements in Mastigophora, 327
Entamoeba, 361
coli, life cycle, 340, 341
mode of life, 340
morphology of, 329, 331, 340, 341
histolytica, 340
as tissue parasite, 327
cultivation of, 339
pathogenicity of, 341
Entero-hepatitis, of turkeys, 372
"Amoeba meleagridis" in, 361
organism of, 372
Entodinium, 352
Enzymes produced by bacteria, 15
Eosin, 358
Eosin-methylene-blue medium, 58
Epidermophyton gallinarum, 314
Epizootic lymphangitis, equine, organism of, 305
Equine infectious anemia, virus of, 408
Equivalent focal distance, defined, 25
Erdmann, 351
Erlert, 203
Erwin, 81
Erysipelas, swine, organism of, 258
Erysipelothrix, 258
genus, specifications of Com. Soc. Amer. Bac., 258
murisepticae, 262
similarity to Erysipelothrix rhusiopathiae, 260
rhusiopathiae, 258
carriers of, 259
cultural characteristics of, 260
immunization against, 261
morphology and staining of, 260
pathogenicity of, 261
resistance of, 261
serological reactions, 261
similarity to Erysipelothrix murisepticae, 260
susceptibility of man to, 259
Erythrocytes, in hemoglobinuria, 349
Escherich, 152
Escherichia, 152
acidi-lactici, 156
coli, 153
cultural characteristics of, 154
immunization against, 155
morphology and staining of, 153
pathogenicity of, 155
resistance of, 155
communior, 155
genus, specifications of the Com. Soc. Amer. Bact., 152

Escherichia group, presence in water, tests for, 493
Esty, 234
Eubacteriales, general description of, 16
Evans, Alice, 176
Evans, Griffith, 6, 362
Evolution, in Trichomonas, 345
Excretory vacuole, in protozoa, 328
Exogenous infection, defined, 102
Exogenous phase, in Coccidia, 347
of Isospora, 347
Exotoxin, in infection, 105
Eye, sarcosporidiosis of, 351

Facultative pathogenic bacteria, defined, 101
Family, Achromatiaceae, general description of, 17
Actinomycetaceae, general description of, 17
Bacillaceae, general description of, 17
Bacteriaceae, general description of, 17
Beggiatocaceae, general description of, 17
Chlamydobacteriaceae, general description of, 17
Coccaceae, general description of, 17
Mycobacteriaceae, general description of, 17
Myxobacteriaceae, general description of, 17
Nitrobacteriaceae, general description of, 17
Rhodobacteriaceae, general description of, 17
Spirillaceae, general description of, 17
Spirochaetaceae, general description of, 17
Fantham, 381
Farcin du boeuf, 251
Farcy, bovine, organism of, 251
equine, mallein tests in, 294
equine, organism of, 289
Favus, canine, organism of, 315
chicken, organism of, 314
fungi causing same, 313
in cats, organism of, 314
mouse, organism of, 315
Feces, sources of error in study of, 337
Fehleisen, 127
Ferry, 183
Feser, 213
Fildes, 76
Filterable virus, 393
defined, 393
of African Horse Sickness, 414
immunization against, 414
resistance of, 414
of aphthous fever, 410
resistance of, 411
of avian diphtheria, 416
resistance of, 417
of bovine contagious pleuro-pneumonia, 404
cultivation of, 404
immunization against, 405
morphology of, 404
resistance of, 405
of canine distemper, 411
resistance of, 412
of equine infectious anemia, 408
carriers of, 409
immunization against, 409
resistance of, 409
of equine influenza, 407
immunization against, 408
transmission of, 408
of foot-and-mouth disease, 410
of fowl diphtheria, 416
of fowl pest, 415
of fowl pox, 416
immunization against, 417
of hog cholera, 405
immunization against, 406
resistance of, 406
of "pestis equorum," 414
of pox in lower animals, 417
of rinderpest, 412
immunization against, 413
resistance of, 412
Filters, bacteriological, 37
cleaning of, 38
method of using, 39
Fischer, 16
Fission, in protozoa, 325
Fixation, of protozoa, 356
"Fixed" rabies virus, 401
Flagella, amphitricha, 9

Flagella general description of, 9
lophotricha, 9
monotricha, 9
peritricha, 9
stains, 95
Flagellates, amoeboid stage in life cycle, 325
euglenoid movement in, 327
formation of food vacuoles, 329
intestinal, pathogenicity of, 345
metabolic form changes in, 327
see Mastigophora
structure of ectoplasm, 327
Flagellum (in protozoa), 328, 341
in Trypanosoma, origin of, 342
relation to basal granule, 333
staining of, 359
Fleming, 81
Flügge, 16, 106
Fodor, 106
Food infection, Salmonella aertrycke in, 160
Salmonella enteritidis in, 160
schottmülleri in, 157
Food intoxication, Clostridium botulinum in, 229
Food-poisoning, Clostridium botulinum in, 229
Salmonella aertrycke in, 160
enteritidis in, 160
schottmülleri in, 157
Food vacuoles, in Protozoa, 329
Foot-and-mouth disease, virus of, 410
"Foot rot," organism of, 253
Forage-poisoning, equine, Clostridium botulinum in, 229
Fowl cholera, organism of, 194
Fowl pest, virus of, 415
Fowl plague, virus of, 415
Fowl pox, immunization against, 417
virus of, 416
Fowl typhoid, organism of, 168
Fractional sterilization, 33
Francis, 198
serum-glucose-cystine agar, 64
Franklin, 218, 474
Frosch, 410
Frothingham, 280
Fumigation, 39
Fungi, 299
classification of, 302
genus Achorion, 313
Aspergillus, 315
Blastomyces, 305
Fusarium, 319
Oidium, 317
Sporotrichum, 318
Trichophyton, 309
imperfecti, 317
Fusarium equinum, 319

Gabbett, 270
method for staining acid-fast bacteria, 90
Gaffky, 219, 220
Gallagher, 361
Galli-Valerio, 380
Gall sickness, bovine, organism of, 384
Galtier, 393
Galziekte, bovine, organism of, 384
Gamaleia, 146, 265
Gambia fever, trypanosome of, 369
Gamete, of Babesia, 350
Gametes, (in protozoa), 333, 345
of coccidia, 347, 348
Gametocytes, of coccidia, 348
Gangrene, gas, organism of, 237
udder of cows, organism of, 253
Gangrenous dermatitis, equine, organism of, 253
Carget, streptococcus of, 135
Gärtner, 160
bacillis, bacillus, 160
Gas bacillus, 237
Gases produced by bacteria, 15
detection of, 81
Gas gangrene, organism of, 237
Gastric vacuoles, in Protozoa, 329
Gastromycosis ovis, organism of, 219
Gatenby, 334
Gay, 449
Gelatin, nutrient, 57
Generic names, rule for, 22
Genus, Actinobacillus, 242
Actinomyces, 244
Aerobacter, 156
Alcaligines, 175
Bacillus, 201

Genus, Borrelia, 295
Clostridium, 213
Corynebacterium, 285
Eberthella, 171
Erysipelothrix, 258
Escherichia, 152
Mycobacterium, 263
Pasteurella, 186
Pfeifferella, 289
Pseudomonas, 149
Salmonella, 157
Staphylococcus, 141
Streptococcus, 127
Vibrio, 146
Gessard, 149
Giardia, 326, 342
Gibson, 480
Giemsa's blood stain, 92
Glanders, 289
agglutination test for, 444
complement-fixation test for, 424
conglutination test for, 449
equine, organism of, 289
mallein tests in, 294
precipitation test for, 448
Strauss test in, 293
Glassware, cleaning of, 40
preparation of for bacteriological use, 40
sterilization of, 33
Goat-pox, virus of, 417
Gochenour, 191, 475
Gonder, 382, 383
Graham, Robert, 229, 230
Gram negative organisms defined, 85
positive organisms defined, 85
Gram's iodine solution, 87
method of staining, 87, 88
method of staining capsules, 95
Granules, basal, in Protozoa, 328
metachromatic, 10
metaplastic, in Trypanosoma, 343
Graybill, 373
Gregarines, 345
Gruber, 112
Gruby, 362
Gugliemi, 377
Guinard, 286
Hadley, 171, 172, 174, 282, 344, 361, 372, 373, 386
Hammer, v
Hanging drop preparation, 83
Haptines of Ehrlich's First Order, 113
of Ehrlich's Second Order, 115
of Ehrlich's Third Order, 116
Harris, 402
Harrison, v
Harris' rabies vaccine, preparation of, 468
Hartmann, 332
Harvey, 167
Harz, 244
Haslam, 218, 474
Hastings, 282
Haughwout, vi, 323, 340, 345, 352, 353, 354, 358, 389, 390
Hay bacillus, 201
Hayem, 107
solution, for red cell counts, 456
Helber, 465
Helman, 294
Hematology, 451
Hematoxylin, Delafield's, formula, 358
Delafield's, Haughwout's modification, 358
Heidenhain's, formula and method, 358
iron-alum, 358
stain of metaplasm in Trypanosoma, 343
Hemoflagellates, identification of, 334
Hemoglobin, content of blood of normal animals, 460
methods for estimating, 458
Hemoglobinophiles, defined, 13
Hemoglobinuria, bovine, organism of, 374
Hemogregarina, fixation and staining of, 356
Hemolysin, Ehrlich's theory of production, 116
in infection, 105
production of, by bacteria, 15
production of, by staphylococci, 142
production of, by streptococci, 129
Hemolysis, defined, 117
Hemolytic amboceptor, in complement-fixation test, 422
Hemolytic system, in complement-fixation test, defined, 423

"Hemorrhagic disease," organism of, 239
Hemorrhagic septicemia, 186
  aggressin, preparation of, 475
  bovine, organism of, 188
  group of organisms, 186
  of buffalo, organism of, 195
  of hogs, organism of, 192
  vaccines, preparation of, 473
Hemosporidia, 346, 349
  cultivation of, 338
  hemozoin pigment in, 329
  identification of, 334
  nuclear division in, 330
Hemozoin, 329
Henley, 478
Hermann's method for staining acid-fast bacteria, 90
Herpetomonad stage, of Trypanosoma, 344
Herpetomonas, 342
Hesse, 241
Hibler's brain medium, 63
Hiss serum-water medium, 55
Hiss's method for staining capsules, 94
Hitchens' agar, one-tenth per cent, 63
Hoffman, 298
Hog cholera, Borrelia hyos in, 298
  immunization against, 406
  Pasteurella suiseptica in, 192
  Salmonella suipestifer in, 161
  "serum alone method" of immunization against, 406
  serum, preparation of, 477
  "simultaneous method" of immunization against, 407
  Spirochaeta hyos in, 298
  virus of, 405
Hogue, 359
Högyes, 402
  rabies vaccine, preparation of, 468
Holman, 128
Holozoic protozoa, 335
Horse-pox, virus of, 417
Host, definitive, 337
  intermediary, 337
  invertebrate, development of protozoa in, 337
  of protozoan parasites, 335
Hot air sterilizer, 32
Huddleson, 81, 179
Hueppe, 186
Huntoon, v, 63
  method for staining capsules, 95
Hydrogen, production of, with Kipp generator, 73
Hydrogen ion, concentration of, in culture media, 45
  discussion of, 45
Hydrophobia, virus of, 393
Hypersensitiveness, discussed, 119
Hyphae, of Hyphomycetes, 302
Hyphomycetes, 301
  non-septated, 302
  septated, 302

Ictero-hemoglobinuria, organism of, 239
Idiosyncrasies, drug, 123
Immunity, 101
  acquired, 104
  active, 104
  agglutinins, Ehrlich's theory of production, 115
  aggressins, 105
  antibody or humoral theory of, 111
  antitoxins, production of, Ehrlich's theory, 113
  bacteriolysins, Ehrlich's theory of production, 116
  defined, 104
  Ehrlich's side-chain theory of, 112
  hemolysins, Ehrlich's theory of production, 116
  individual, 104
  in Piroplasmosis, 350
  natural, 104
  passive, 104
  phagocytic theory of, 107
  precipitins, Ehrlich's theory of production, 115
  racial, 104
  side-chain theory of, Ehrlich's, 112
  specific, 104
  theories of, 106
  types of, 104
India ink method for staining capsules, 95
Indicators, colorimetric, 47
Indol, tests for, 53

Infection and Immunity, 101
theories of, 105
Infection, defined, 101
endogenous, defined, 102
exogenous, defined, 102
Infectious leukemia of fowls, organism of, 169
Inflammation, in protozoal infection, 335
Influenza, equine, immunization against, 408
virus of, 407
Infusoria, 326, 389
characters of, 352
ectoplasmic modifications in, 328
formation of food vacuoles, 329
reproduction in, 334, 352
see Ciliates
stomachs of ruminants, in, 352
structure of nuclei, 331
Ingram, 281
Inoculation of culture media, methods, 65
Iodamoeba büstchlii, nuclear plastin, 331
Iodine, Weigert for cysts, 359
Iron-alum, 358
Isospora, 346, 388
bigemina, 347, 388
appearance of cysts in feces, 347
development in host, 347
distinction from Eimeria, 348
life cycle, 347–349
see also Eimeria, Coccidia and Coccidium

Janicki, 332
Janssen, 3
Janus Green "B," for staining flagella of protozoa, 359
Jenner, 6, 418
Johne, 280
Johne's bacillus, 280
disease, allergic tests for, 282
organism of, 280
"Johnin," 282
Jones, 167, 447

Kalning, 294
Karyogamy, in protozoa, defined, 333
Karyokinesis, in protozoa, 330
Karyosome, in nucleus of Trypanosoma, 342
structure, 331
"Keel" in ducklings, organism of, 165
Kekule, 113
Kelser, 235, 340, 343, 353, 395
Kent, 362
Kessel, 345
Kilborne, 7, 374, 375
Kinetonucleus, 330, 332, 342
King, 298
Kipp generator for hydrogen production, 73
Kirkpatrick, 167
Kitasato, 7, 111, 195, 214, 224
Kitt, 188, 218
Klein, 168, 171
Klencke, 264
Koch, 5, 6, 203, 219, 220, 264, 382
Kofoid, 342
Kolle, 413
Kraus, 112
Krumwiede and Pratt's method of cultivating anaerobes, 76

Lactose-bile medium, 55
Ladson, 176
Laidlaw, 76
Laveran, 6
Leclainche, 262
Lee, 334
Leeuwenhoek, 3
Lehmann, 16
Leishman, 108, 109, 371
Leishmania, 342, 371
canis, 372
cultivation of, 338
donovani, 372
demonstration of, in tissues, 371
infantum, 371
Leishmaniasis, canine, organism of, 372
Lemaistre, 168
Lens, homogeneous oil immersion, 29
Leprosy, 284
Leucocidin in infection, 105
method for demonstration of, 131
production of, by staphylococci, 142
production of, by streptococci, 130

Leucocytes, differential counts, technic of 459
technic of counting, 457
Leukemia, infectious, of fowls, organism of, 169
Levy, 453
Lewis, 362
Ligineres, 157, 242, 377
"Limberneck," Clostridium botulinum in, 230
Linin, 331
"Lip-and-leg ulceration," of sheep, organism of, 253
Lipases, production of, by bacteria, 15
Lister, 5
Lithobius forficatus, coccidium of, 346
Litmus milk, 54
Liver broth, 54
Liver, trichomoniasis of, in man, 345
Lizards, coccidial cysts in feces of, 337
Lloyd, 62
Lockjaw, organism of, 223
Loeffler, 162, 192, 253, 258, 289, 410
alkaline-methylene-blue stain, 86
serum medium, 60
"Loffleria nevermanii," see virus of aphthous fever, 410
Lophophyton gallinarum, 314
Lorenz, 262
Lowden, 395
Lubenau's glycerin-egg medium, 59
"Lumpy jaw," organism of, 244
"Lung plague," bovine, virus of, 404
Lymphangitis, epizootic, equine, organism of, 305
farciminosa bovis, 251
Sporotrichum veurmanni in, 318
ulcerative, equine, organism of, 286

Mack, 239
MacNeal, 338, 362
Macrogametes, of coccidia development, 348
Macrophages, defined, 108
Malaria, cultivation of parasite of, 338
Mal de caderas, complement-fixation test for, 436
trypanosome of, 368
Mal du coit, trypanosome of, 366
Malignant edema, organism of, 219
Malignant jaundice, canine, organism of, 380
Malignant pustule, organism of, 203
Mallein, 294, 481
intradermal, preparation of, 483
methods for preparation of, 483
ophthalmic, preparation of, 483
reaction, an allergic manifestation, 123
subcutaneous, preparation of, 483
tests in glanders, 294
Mallory's staining method for bacteria in tissue, 93
Malmsten, 353
Malta fever, 180
agglutination test for, 446
complement-fixation test for, 439
organism of, 180
Mann's stain for Negri bodies, 92
Marchoux, 208, 296, 297
Markus, 281
Marmock, 129
Martin's peptone broth, 52
Martoglio, 297
Marx, 417
Mastbaum, 262
Mastigophora, 326, 341, 362
amoeboid stage in life cycle, 325
division in, 334
euglenoid movement in, 327
structure of ectoplasm, 327
undulating membrane, 327, 342
Mastitis, bovine, streptococcus of, 135
Mazureano, 381
McBryde, 406
McCoy, 196, 197, 198
McDonald, 353, 354, 355, 390
McFadyean, 281, 414
McIntosh, 76
McIntosh and Fildes' method of cultivating anaerobes, 76
Meat mash medium for anaerobes, 63
Mediterranean fever, organism of, 180
Meiosis, in coccidia, 348
Melvin, 319
Membrane, undulating, of flagellates, 327, 342

Membrane, of Infusoria, 328, 352
of Trypanosoma, 342
Membranelles, 328, 352
Merozoite, development to gametocyte, 348
of coccidia, 348
Metabolic movement, in Mastigophora, 327
Metaplasm, in Protozoa, 329, 343
Metchnikoff, 106, 107, 108
Methods, bacteriological, 23
Meyer, 227, 234, 239, 281, 382, 441
M'Gowan, 183
Microaerophiles, defined, 13
Micrococcus melitensis, 180
Microgamete, of coccidia, 348
Microgametocytes, of coccidia, 348
Microorganisms and disease, 101
Microphages, defined, 108
Microscope, 25
condenser, description of, 28
dark field examinations, method of making, 29
early history of, 3
magnification table, 27
mirrors, description of, 28
oculars, description of, 27
oil immersion lens, described, 29
tube length, discussion of, 28
use and care of, 30
Microscopic study of bacteria, 83
Microsporum, 312
audouini, variety canis, 312
felinum, 313
lanosum, 312
minimum, 312
Miescher's sacs or tubes, 351, 388
Migula, 16
Milk, 487
bacterial counts, plating method, 487
Breed direct microscopic method, 489
certified, 490
collection of samples for bacteriological examination, 488
pasteurization of, 491
Streptococcus lactis in, 140
whey medium, 54
Minchin, 333, 359, 370
Mirrors, microscope, description of, 28
Mitosis, in protozoa, 330
Mitzmain, 364
Moeller's method of staining spores, 98
Mohler, 286, 319, 437
Monilia albicans, 317
candida, 317
Moore, 169
Morphology, of bacteria, 8
Motility, 83
Brownian, 83
determination of, 83
Moulds, pathogenic, 301, 309
Mouse typhoid, Salmonella typhi-murium in, 162
Mudge, 136
Mueller, 15
Muir's method of staining capsules, 94
modification of Pittfield's method for staining flagella, 95
Murrina, trypanosome of, 368
Mycelium, of Hyphomycetes, 302
Mycobacteriaceae, general description of, 17
Mycobacterium, 263
avium, 279
cultural characteristics, 280
morphology and staining, 280
pathogenicity, 280
butyricum, 283
genus, specifications of Com. Soc. Amer. Bact., 263
leprae, 284
paratuberculosis, 280
biproducts of, 282
cultural characteristics, 281
morphology and staining, 281
pathogenicity, 282
resistance of, 282
serological reaction, 283
phlei, 283
smegmatis, 283
tuberculosis, 263
biological therapy and prophylaxis, 279
cultural characteristics, 271
difference between bovine and human types, 274

Mycobacterium, tuberculosis, Gabbett's method of staining, 270
Hermann's method of staining, 270
morphology and staining, 269
Pappenheim's method of differentiating from Mycobacterium smegmatis, 270
pathogenicity of, 278
resistance of, 278
serological reaction, 279
toxic products of, 275
types of, 265
Ziehl-Neelsen's method of staining, 270
Mycomycetes, 302
Fungi imperfecti, 317
Myoneme fibres, in Mastigophora, 327
Myxobacteriaceae, general description of, 17
Myxobacteriales, general description of, 16

Nagana, 365
complement-fixation test for, 436
trypanosome of, 365
Nageli, 16
Nakayama, 130, 131
Names, family, ending of, 17
generic, rule for, 22
order, ending, of, 16
sub-family, ending of, 22
rule for, 22
tribe, ending of, 22
Necrobacilloses, organism of, 253
Needham, 4
Negative phase, defined, 109
Negri, 394, 395
bodies, 394
methods of demonstrating, 396
nature of, 395
staining of, 92
Neisser's stain for polar bodies, 89
Neosporidia, 346
Neubauer, 454
"Neurocytes hydrophobiae," in rabies, 395
Neuromotor system, in protozoa, 324, 333, 342
Newmann, 16
Nicolaier, 6, 224
Nicolle, 362, 371, 412
Nicolle, Novy, MacNeal medium, 63
Niles, 406
Nitrites, tests for, 53
Nitrobacteriaceae, general description of, 17
"N.N.N." medium, 63
Nocard, 163, 251, 286, 404, 414
Nocardia farcinica, 251
Noguchi, 75
Nörgaard, 218, 286, 319
Nosema, germinal transmission of, 338
Novy, 338, 362
Nuclear reduction, in coccidial gametes, 348
Nuclei, dimorphic in ciliates, 330, 352
Nucleoplasm, in protozoa, 326
Nucleus, division in Haemosporidia, 330
Karyosome type in protozoa, 331
in trypanosomes, 342
of protozoa, 329
protozoan, staining of, 357
pyknotic, 331
"ring" type in protozoa, 330
study, of in relation to protozoan physiology, 329
Numerical aperture, defined, 28
Nuttall, 106, 111, 237
Nuttallia equi, 377
Nutrition, in protozoa, 335
Nutritional requirements of bacteria, 11

Objectives, microscope, 25
parfocal, 27
Oculars, microscope, 27
Oidium, 317
albicans, 317
Oöcyst, formation of in coccidia, 348
Oökinete, of Babesia, 350
Oöspora canina, 315
Ophryoscolex, 353
Opsonic index, defined, 109
Wright's method for determining, 109
Opsonin, defined, 108
Oreste, 195
Organelles, behavior in dividing protozoön, 330
Orla-Jensen, 16
Order, Actinomycetales, general description of, 16

Order, Chlamydobacteriales, general description of, 16
Eubacteriales, general description of, 16
Myxobacteriales, general description of, 16
Spirochaetales, general description of, 16
Thiobacteriales, general description of, 16
Ostertag, 409
Ovum, homology with macrogamete, 348
Oxygen displacement method of cultivating anaerobes, 72
exclusion method of cultivating anaerobes, 72
exhaustion method of cultivating anaerobes, 72

Panum, 107
Pappenheim's staining method for differentiating C. tuberculosis and C. smegma, 91
Parabasal body, 330
in Trichomonas, 332
in Trypanosoma, 342
Parasite, lethal, 338
Parasitic bacteria, defined, 101
non-pathogenic, defined, 101
Parasitism, adaptation of protozoa to, 326
development of, in protozoa, 335, 336
Paratubercular dysentery, bovine, organism of, 280
Paratuberculous enteritis, bovine, organism of, 280
Paratyphoid fever, organism of, 157
Parfocal objectives, 27
Pasteur, 4, 6, 194, 209, 219, 220, 261, 394, 402, 470
anthrax vaccine, preparation of, 470
"treatment" for rabies, 466
vaccine for anthrax, 209
Pasteurella, 186
avicida, 194
cultural characteristics, 194
morphology and staining, 194
bubalseptica, 195
boviseptica, 188
cultural characteristics of, 189
immunization against, 190
morphology and staining of, 188
pathogenicity of, 190
resistance of, 190
equiseptica, 191
immunization against, 192
morphology and staining of, 192
genus, specifications of Com. Soc. Amer. Bact., 186
muriseptica, 262
pestis, 195
carriers of, 196
cultural characteristics of, 197
morphology and staining of, 196
suiseptica, 192
cultural characteristics of, 193
morphology and staining of, 193
tularensis, 197
carriers of, 198
cultural characteristics of, 199
medium for, 64
morphology and staining of, 199
pathogenicity of, 200
serological reaction, 200
Pasteurization of milk, 491
Pathogenic bacteria, defined, 101
Pathogenic fungi, 299
Paulicki, 265
Pearson, 230
Peptone solution, Dunham's, 53
Peripheral chromatin, 331
"Pernicious anemia," equine, virus of, 408
Perroncito, 6, 194, 415
Pestis equorum, virus of, 414
Petroff's medium, 59
Pfaff, 173
Pfeiffer, 106, 111, 116
Pfeifferella, 289
genus, specifications of Com. Soc. Amer. Bact., 289
mallei, 289
cultural characteristics of, 292
morphology and staining of, 292
pathogenicity of, 293
resistance of, 293
serological reactions, 293
Strauss test for identification of, 293
Pfeiler, 448
pH, defined, 46

Phagocytes, fixed, 107
motile or wandering, 107
Phagocytic index, defined, 110
Phagocytosis, defined, 107
Phenomenon of Arthus, 120
Photosynthesis, 324
Phycomycetes, 302
Piana, 380
Pig, balantidia of, 353
sarcosporidiosis of, 351
Pigment, 15
of Pseudomonas aeruginosa, 151
of staphylococci, 141
"Pink eye," equine, virus of, 407
Pipettes, preparation and sterilization of, 41
Piroplasma, 346, 374
bigeminum, 374
caballi, 377
canis, 380
development in ticks, 349
equi, 377
fixation and staining of, 355
life history, 349
mutans, 379
nuclear division in, 330
ovis, 381
see also Babesia
systematic position, 349
transmission, 349
Piroplasmoses, organisms of, 374
Piroplasmosis, bovine, 351, 374
canine, 350, 380
equine, 377
geographical distribution, 349
host reactions, 350
immunity in, 350
Pitfield's method (Muir's modification) for staining flagella, 95
Plague, bubonic, organism of, 195
Plasmodium, 346
cultivation of, 338
fixation and staining of, 355
nuclear division in, 330
toxins in, 336
Plasmogamy, see plastogamy
Plasmosome, 331
Plastin, 331
Plastogamy, in protozoa, 334
Plates, pour, 68
streak, methods of inoculating, 69
Plating, methods of 68
Platinum loops and needles, 66
Plenciz, 4
Pleomorphism of bacteria, 9
Pleuro-pneumonia, contagious, bovine, virus of, 404
equine, Pasteurella equiseptica in, 191
Streptococcus equi in, 133
Pneumomycosis, organisms causing, 315
Pneumonia, mycotic, Aspergilli in, 315
Poels, 407, 408
Pollender, 5, 203
Poor, 394
Pork broth, 54
Potato, media, 54, 59, 60
Pour plates, 68
Pox, 417
bovine, virus of, 417
equine, virus of, 417
fowl, immunization against, 417
virus of, 416
goat, virus of, 417
sheep, virus of, 417
swine, virus of, 417
Precipitation test, for anthrax, 448
for glanders, 448
for swine erysipelas, 449
Precipitins, 115
production of, Ehrlich's theory, 115
theoretical structure of, 116
Preisz, 218, 286
Preisz-Nocard bacillus, 286
Principal focal distance, defined, 27
Pronuclei, in coccidia, 348
Propagative phase, in coccidia, 347
Proteases, production of by bacteria, 15
Proteolysis, by protozoa, 336
Protophyta, 324
Protozoa, 323
alternation of generations in, 337
of hosts in, 337
axioms in study of, 360
binary fission in, 325
blood, 346
carnivorous, 335

Protozoa, cellular and tissue reactions to, 360
colonial forms, 324
compared with metazoa, 323
cultivation "in symbiosis" with bacteria, 339
cysts of, 337
cytological technique, 334
degeneration in, 323, 334, 355
developmental cycles in, 337
development of parasitism in, 336
digestion in, 335
fallacies in study of, 323, 325, 327, 329, 330, 332, 333, 334, 335, 336, 337, 338, 344, 346, 351, 355, 360
food, choice of by, 339
form changes in life cycle, 325
free-living, cultivation of, 338
holozoic, 335
inflammatory reaction with, 335
intestinal, of man, cultivation, 339
life cycle, 324
metaplastic bodies in, 329
method of attack on tissues, 336
morphology of, 326
new species of, 360
non-cellular organisms, 324
pathogenicity of, 335, 360
physiology of, 335
potential germ cells, 325
pure cultures of, 339
rejuvenescence in, 325
relation to toxaemia, 335
reproduction in, 325
"saprophytic," 335
saprozoic, 335
sexual phenomena, in, 333, 360
staining methods, 357
structure of ectoplasm, 327
structure of the nucleus, 329
syngamy in, 333
technical methods, 355
tissue destruction by, 335, 336
tissue reactions produced by, 335
toxins of, 335
transmission of, 337
vegetarian, 335
Prowazek, 418
Pseudomonas, 149
aeruginosa, 149
cultural characteristics of, 150
morphology and staining of, 150
pigments of, 151
production of pyocyanase by, 151
resistance of, 151
toxin of, 151
genus, specifications of Com. Soc. Amer. Bact., 149
Pseudopodia, 328, 339
Pseudotuberculosis of sheep, organism of, 286
Pure cultures, methods of obtaining, 68
Pus, green, organism causing, 149
Pyocyanase, production of by pseudomonas aeruginosa, 151
Pyocyanin, production of by Pseudomonas aeruginosa, 151

Quarter-evil, organism of, 213
Quarter-ill, organism of, 213

Rabbit coccidiosis, organism of, 386
"Rabbit fever," organism of, 197
Rabbit "snuffles," Alcaligines bronchisepticus in, 184
Rabbits, coccidiosis in, 346
Rabies, 39
animal inoculations, methods of, 400
antirabic serum in treatment of, 403
diagnosis of, 395
dissemination of the infection, 401
immunization, 401
Cumming's method, 403
Harris' method, 402
Högyes' method, 402
Pasteur's method, 401
single injection method, 403
Umeno & Doi's method, 403
Negri bodies in, description of, 394
methods of demonstrating, 396
nature of, 395
"street" and "fixed" viruses defined, 401
vaccine, 468
Cumming method of preparation, 470
dose schedule for Pasteur vaccine, 468
Harris, method of preparation, 468

Rabies, vaccine, Högyes, preparation of, 468
technic of Pasteur's method of preparing, 466
Umeno & Doi's method of preparation, 470
virus, 393
dissemination of, 401
experimental inoculation of, methods, 400
resistance of, 401
Rainey's corpuscles, 351
Ransom, 227
Rattone, 223
Rat, trypanosome of, 344
Rayer, 5
"Ray fungus" of Actinomycosis, 244
Receptors, discussed, 114
Records, 239, 240, 241
"Red dysentery," bovine, organism of, 387
"Red fever," swine, organism of, 258
"Red water," bovine, organism of, 374
"Red water disease," organism of, 239
Rejuvenescence in Uroleptus mobilis, 325
Remlinger, 394
Reproduction, in Babesia, 350
in Gregarines, 345
in Infusoria, 352
in parasitic protozoa, 337
in Sporozoa, 345
in Trypanosoma, 344
of Entamoeba coli, 341
Resistance, 101
Rettger, 165, 167, 174
Reynolds, vi, 437
Rhipicephalus sanguineus, 350
Rhizopoda, 339
Rhodesian red-water, organism of, 382
tick fever, organism of, 382
Rhodobacteriaceae, general description of, 17
Richet, 119, 120
Rinderpest, 412
serum, preparation of, 479
vaccination against, 413
virus of, 412
Ringworm, 309
bovine, organisms of, 311, 312
Ringworm, canine, organisms of, 311, 312, 313
equine, organisms of, 311, 312
feline, organisms of, 312, 313
of fowls, organism of, 312
Rivolta, 265, 305
Roderick, 408
Rosenau, 120
Rosenbach, 6, 127
Ross, 371
Rouget du porc, organism of, 258
Roux, 209, 213, 394, 404
Ruediger, 130
Ruget, 366
Rupp, 136, 137
Russell's double sugar medium, 61

Sabouraud's medium, 57
Saccharomyces albicans, 317
farciminosus, 305
Sahli's method of estimating hemoglobin, 458
Sakharoff, 295
Salimbeni, 296, 297
Salmon, 157, 161
Salmonella, 157
abortus-equi, 163
immunization, 165
morphology and cultural characteristics, 164
aertrycke, 160
anatum, 165
cultural characteristics of, 166
morphology and staining of, 166
pathogenicity of, 167
enteritidis, 160
carriers of 161
gallinarum, 168
cultural characteristics of, 169
morphology and staining of, 169
genus, specifications of Com. Soc. Amer. Bact., 157
paratyphi, 170
morphology and cultural characteristics, 170
psittacosis, 163
pullorum, 167
carriers of 167

Salmonella psittacosis, morphology and cultural characteristics, 167
technic of agglutination test for carriers of, 447
schottmülleri, 157
cultural characteristics of, 158
immunization, 160
morphology and staining of, 158
resistance of, 160
suipestifer, 161
typhi-murium, 162
Salsbery, 474
Sap, nuclear, see enchylema, 331
Saprophytes, defined, 101
"Saprophytic" protozoa, 335
Saprozoic protozoa, 335
Sarcocystis, 351, 388
anatomical distribution, 351
bertrami, 388
blanchardi, 388
developmental cycle of, 351
duration of life, 351
in intestinal wall, 351
in skeletal muscles, 351
miescheriana, 388
morphology, 351
tenellae, 388
toxins in, 336
Sarcodina, 326, 339, 361
flagellated stage in life cycle, 325
see amoebae
structure of ectoplasm, 327
Sarcosporidiosis, 351, 388
artificial production, 351
fecal infection in, 351
of domestic animals, 351, 388
of man, 351
Savonuzzi, 415
Schalk, 408
Schaudinn, 325, 346, 354, 357
Schizogony, in coccidia, 348
in protozoa, 325
in Trypanosoma, 344
Schizomycetes, general description of, 16
Schizont, of coccidia, 348
Schneider, 141
Schöbl, 218
Schoening, 403, 437
Schottmüller, 127
Schroeder, 4, 177, 180
Schultz, 122, 192
Schütz, 132, 262, 289
Schwann, 4
Sclavo, 208
Scoville, 165
Senility, in protozoa, 325
Septicemia of mice, organism of, 262
Sera, preparation of, 476
Serological tests, methods for, 421
Serology, 419
Serum, anti-anthrax, preparation of, 476
anti-bacterial, preparation of, 479
anti-hog-cholera, preparation of, 477
anti-rinderpest, preparation of, 479
anti-staphylococcic, preparation of, 479
anti-streptococcic, preparation of, 479
broth, 56
Sharp, 355
Sheep-pox, virus of, 417
Shipping-fever, equine, virus of, 407
Smith, 128
Smith, Theobald, 7, 75, 120, 161, 249, 264, 274, 361, 372, 374, 375
Smith tubes, use of, 81
"Snuffles," rabbit, Alcaligines bronchisepticus in, 184
Sobernheim, 210
Sorensen, 46
Sorillon, 251
Souma, trypanosome of, 369
Spallanzani, 4
Spermatozoon, homology with microgamete, 348
Spirilla, general description of, 8
Spirillaceae, general description of, 17
Spirillum anserina, 295
ovis, 297
theileri, 297
Spirochaeta equi, 297
gallinarum, 295
hyos, 298
ovis, 297
theileri, 297
Spirochaetaceae, general description of, 17
Spirochaetales, general description of, 16

Spirochaetes, relation to Cyanophaceae, 342
relation to Mastigophora, 342
Spitz, 242
Splenic fever, organism of, 203
Spontaneous generation theory, 4
Sporangium, 304
Spores, 10
formation, in Sporozoa, 345
general description of, 10
in Coccidia, encystment of, 347
methods for staining, 97
Sporoblast, 349
of coccidia, formation, 349
of Eimeria, formation, 349
of Isospora, formation, 349
Sporocyst, formation in Eimeria, 349
of coccidia, formation, 349
of Isospora, formation, 349
Sporogony, in coccidia, 349
in protozoa, 325
Sporotrichosis, equine, 318
Sporotrichum, 318
schencki, 319
veurmanni, 318
Sporozoa, 326, 345, 374
ectoplasm in, 328
gametes of, 345
reproduction in, 345
Sporozoites, of coccidia, 347, 349
Spreaders for inoculating culture media, 67
Stab cultures, 70
Staining, 84, 357
bacteria, 84
protozoa, 357
reactions in nucleus, 329
Stains, 86
Abbott's method for spores, 97
acid-fast bacteria, methods, 89
alkaline-methylene-blue (Loeffler's), 86
aniline-gentian-violet, 87
aniline-gentian-violet (Stirling's), 88
blood, Giemsa, 92
Wright, 91
capsule, Gram's method, 95
Hiss's method, 94
Huntoon's method, 95
India ink method, 95
Muir's method, 94
Welch's method, 93
carbol-fuchsin (Ziehl-Neelsen), 86
cytoplasmic, 358
flagella, 95
Pitfield method (Muir's modification), 95
Van Ermengem's method, 96
Zettnow's method, 97
for Negri bodies, 92
Gabbet's for acid-fast bacteria, 90
gentian-violet, Weigert's, 88
Giemsa's, blood, 92
Gram's iodine solution, 87
Gram's method, 87, 88
hematoxylin, Delafield's, etc., 358
Hermann's method for acid-fast bacteria, 90
Hiss's method for capsules, 94
Huntoon's method for capsules, 95
India ink method for capsules, 95
Loeffler's alkaline-methylene-blue, 86
Mallory's for bacteria in tissue, 93
Mann's for Negri bodies, 92
Moeller's method for spores, 98
Muir's method for capsules, 94
Neisser's for polar bodies, 89
Pappenheim's method for differentiating C. tuberculosis and C. smegma, 91
Pitfield method (Muir's modification) for flagella, 95
spore, 97
Abbott's method, 97
Moeller's method, 98
Stirling's aniline-gentian-violet, 88
Van Ermengem's method for flagella, 96
Van Gieson's for Negri bodies, 92
Weigert's gentian-violet, 88
Williams' modification of Van Gieson's method for Negri bodies, 93
Wright's, blood, 91
Zettnow's method for flagella, 97
Ziehl-Neelsen's carbol-fuchsin, 86
method for acid-fast bacteria, 90
Staphylococci, immunity to, 142
pigments of, 141
Staphylococcus, 141
albus, 145

Staphylococcus, aureus, 142
cultural characteristics of, 143
morphology and staining of, 142
resistance of, 144
citreus, 145
genus, specifications of the Com. Soc. Amer. Bact., 141
hemolysin, 142
leucocidin, 142
pyogenes albus, 145
aureus, 142
citreus, 145
serum, preparation of, 479
Steinhardt, 394
Sterilization, 31
by autoclave, 33
by boiling, 33
by chemicals, 39
by filtration, 36
by flowing steam, 33
by hot air, 32
by steam under pressure, 33
fractional, 33
of glassware, 33
temperatures obtained with steam under pressure, 36
through use of gases, 39
Sternberg, 16
Sticker, 417
Stirling's aniline-gentian-violet stain, 88
Stomatitis, necrotic, of swine, organism of, 253
thrush-like, Oidium albicans in, 317
Stordy, 297
Strangles, equine, organism of, 132
Strauss, 265
test in glanders, 293
Streak plates, methods of inoculating, 69
"Street" virus of rabies, 401
Streptococci, alpha type, 128
beta type, 128
biproducts of, 129
classification of, 127
gamma type, 128
immunity to, 132
of animal origin, pathogenicity for man, 129
Streptococcus, 127
agalactiae contagiosae, 135
equi, 132
cultural characteristics of, 133
in equine contagious pleuro-pneumonia, 133
morphology and staining of, 133
pathogenicity of, 135
resistance of, 135
genus, specifications of Com. Soc. Amer. Bact., 132
hemolysin, 129
hemolyticus, 127
lactis, 140
leucocidin, 130
mastitidis, 135
carriers of, 138
cultural characteristics of, 135
differentiation from Streptococcus pyogenes, 136
"final pH" of cultures of, 136
morphology and staining of, 135
resistance of, 138
mitior, 127
of bovine mastitis, 136
pyogenes, 138
cultural characteristics of, 138
differentiation from Streptococcus mastitidis, 136
morphology and staining of, 138
pathogenicity for lower animals, 129, 138
pathogenicity of, 140
resistance of, 140
serum, preparation of, 479
toxin of, 131
viridans, 127
Streptothrix, 244
bovis, 244
cuniculi, 253
farcini bovis, 251
farcinica, 251
necrophora, 253
Sub-families of bacteria, 22
Sub-family names, ending of, 22
Surra, 362
carriers of organism of, 363
complement-fixation test for, 365, 436
trypanosome of, 362

"Swamp-fever," virus of, 408
Swezy, 332
Swine erysipelas, 258
  organism of, 258
  precipitation test for, 449
  serum treatment of, 262
  vaccination against, 261
Swine plague, organism of, 192
Swine-pox, virus of, 417
Symbiosis, definition of, 13
Symptomatic anthrax 213
  aggressins in immunization, 218
  immunization, 217
  organism of, 213
Syngamy, 334
  in coccidia, 347

Tallquist's method of estimating hemoglobin, 459
Tarozzi, 75
Teague's medium, 58
Technical methods, with protozoa, 355
Telosporidia, 345
Temperature requirements of bacteria, 11
Temperatures, in cultivation of bacteria, 71
Test, agglutination, technic of, 442
  agglutinin absorption, technic of, 447
  Ascoli's, for anthrax, 448
  complement-fixation, description of, 422
  conglutination, technic of, 449
  for acetyl-methyl-carbinol, 54
  for indol, 53
  for nitrites, 53
  precipitation, technic of, 448
  serological, methods for, 421
  Voges-Proskauer, 54
Tetanolysin, production of by Clostridium tetani, 228
Tetanus, 223
  organism of, 223
  toxin, 227
Tetanus antitoxin, preparation and standardization of, 479
    unit defined, 480
Texas fever,
  bovine, immunization against, 377
    organism of, 374
Thallophytes, 301
Theiler, vi, 297, 370, 378, 379, 382, 383, 384, 385, 414, 415
Theileria, 349, 381
  parva, 350, 382
    life cycle of, 383
    morphology of, 383
    transmission of, 382, 383
  specificity and immunity, 350
Thiobacteriales, general description of, 16
Thoma, 453
Thomas, 213, 217, 218
Thomson, 370, 381
Thrush, in children, organism of, 317
Tick fever, bovine, organism of, 374
Ticks, in bovine piroplasmosis, 351
  vectors of Piroplasmata, 349
Timothy hay bacillus, 283
Tissue enrichment method of cultivating anaerobes, 75
Tissue invasion by Trichomonads, in man, 345
  in turkey, 344
Titration of culture media, 49
Toisson's fluid for red cell counts, 455
Tokishige, 305
Toussaint, 194, 209
Toxemia, in protozoan invasion, 335
Toxin, 15
  diffusible, not produced by protozoa, 335
  in Plasmodium, 336
  of protozoa, 335
  produced by Clostridium botulinum, 233
    by Clostridium chauvei, 216
    by Clostridium hemolyticus bovis, 241
    by Clostridium luciliae, 236
    by Clostridium oedematis-maligni, 223
    by Clostridium tetani, 227
    by Clostridium welchii, 239
    by Pseudomonas aeruginosa, 151
    by Streptococci, 131
  production of by bacteria, 15
  soluble, defined, 15
    in infections, 105
  tetanus, affinity for nerve tissue, 227
    resistance of, 227
  theoretical structure of, 114
  true, defined, 15
Toxoid, defined, 115

Transmutation of bacteria, 9
Traum, 437
Tribe names, ending of, 22
Tribes of bacteria, 22
Trichina, calcification in muscles, 351
Trichomonads, 344, 372
Trichomonas, 342
  adaption to carnivorous diet, 345
  adaption to tissue life, 345
  genus, 344, 372
  in blood stream, 345
  in bowel wall of man, 345
  in bowel wall of turkey, 345
  meleagridis, 372
  morphology, 327, 328, 332, 333
  parabasal body in, 332
  tissue invasion by, 344
  undulating membrane in, 327
Trichophyton, 309
  equinum, 311
  felineum, 312
  granulosum, 311
  megalospora types defined, 309
  megnini, 312
  mentagrophytes, 311
  microspora types defined, 309
  niveum, 312
"Trophonucleus," in Trypanosoma, 332
Trophozoite, of coccidia, 348
Trypanosoma, 342, 362
  agglomeration, 334, 344
  binary fission in, 344
  brucei, 365
    morphology of, 366
    pathogenicity, 366
    transmission of, 366
  carriers, 344, 363, 366, 370
  cazalboui, 369
  character of cytoplasm, 342
  congolense, 370
  crithidial stage, 344
  cruzi, development in lung, 344
  cycle in arthropod host, 344
  development in vertebrate host, 344
  dimorphon, 369
  equinum, 368
    morphology of, 368
    transmission of, 368
  equiperdum, 366
    morphology of, 367
    pathogenicity of, 367
    transmission of, 367
  evansi, 362
    carriers of, 363
    morphology of, 364
    pathogenicity of, 364
    transmission of, 364
  fixation and staining of, 355
  herpetomonad stage, 344
  hippicum, 368
    morphology of, 368
    pathogenicity of, 369
    transmission of, 369
  lewisi, 370
    cultivation of, 338
  morphology, 342
  movement, 342
  of dourine, 366
  of gambia fever, 369
  of mal de caderas, 368
  of murrina, 368
  of nagana, 365
  of souma, 369
  of surra, 362
  of valeri, 369
  pecaudi, 369
  polymorphism in, 342
  reproduction in, 344
  schizogony in, 344
  theileri, 370
Trypanosomiasis, complement-fixation test for, 436
Tubercle bacillus, 263
Tuberculin, 275, 481
  intradermic, 276
    preparation of, 482
  Koch's Old Tuberculin or "O.T.", 276
    preparation of, 481
  ophthalmic, 277
    preparation of, 482
  reaction, an allergic manifestation, 123
  subcutaneous, 277
    preparation of, 481
  test, 276
  toxicity of, 275
Tuberculosis, 263

Tuberculosis, avian, 279
bovine, eradication of, 267
in children, 265
vaccination against, 279
"Bovovaccine" in, 279
free accredited herds, 267
organism of, 263
serological tests in, 279
swine, due to avian type, 266
due to bovine type, 266
due to human type, 266
tuberculin tests in, 276
Tubes, Miescher's, 351, 388
Tularemia, organism of, 197
Tulloch, 229
Turner, 413
Twort, 14, 281
Twort-d'Herelle phenomenon, 14
Typhoid bacillus, 171
Typhoid, fowl, organism of, 168
mouse, Salmonella typhi-murium, 162
Tyzzer, 346

Ulcerative lymphangitis, equine, organism of, 286
Ultramicroscopic filterable viruses, 393
Umeno, 403, 470
Umeno & Doi's method of preparing rabies vaccine, 470
Undulant fever, organism of, 180

Vaccine,
anthrax, 208
Pasteur's method of preparing, 470
spore, 472
blackleg, methods for preparation of, 472
bovine infectious abortion, preparation of, 473
definition of, 463
hemorrhagic septicemia, preparation of, 473
rabies, Cumming method of preparation, 470
preparation according to Harris' method, 468
preparation according to Högyes' method, 468
technic for Pasteur's method of preparing, 466
Umeno and Doi's method of preparing, 470
standardization, methods for, 465
symptomatic anthrax, methods of, 217, 472
swine erysipelas, 261
Vacuoles, as sign of degeneration in protozoa, 329
digestive or gastric, in protozoa, 329
of Entamoeba coli, 340
Valentine, 362
Valeri, 409
Vallee, 409
Van de Velde, 142
Van Ermengem, 229, 233
method for staining flagella, 96
Van Gieson's stain for Negri bodies, 92
Van Helmont, 4
Vanillin test for indol, 53
Vaughan, 121
Vawter, 239, 240, 241
Veal broth, 54
Vectors, arthropod, 337
in trypanosomiasis, 343, 364, 366, 368, 369, 370
Vedder's starch agar medium, 60
Vibrio, genus, specifications of Com. Soc. Amer. Bact., 146
Vibrio metchnikovi, 146
cultural characteristics, 147
morphology and staining, 146
pathogenicity, 148
Vibrio, organisms of the genus, 146
Vibrion septique, 219
Villemin, 264
Virulence, defined, 102
Viruses, filterable, 391
Virus of aphthous fever, 410
of avian diphtheria, 416
of bovine contagious pleuro-pneumonia, cultivation of, 404
of canine distemper, 411
of contagious pleuro-pneumonia, bovine, 404
of equine infectious anemia, 408

Virus of equine influenza, 407
of foot-and-mouth disease, 410
of fowl pest, 415
of fowl pox, 416
of hog cholera, 405
of pox in lower animals, 417
of rabies, 393
of rinderpest, 412
Voges, 262
Voges-Proskauer test, 54
Volpino, 394
von Langenbeck, 244

Water, 491
bacteriological examination of, 491
tests for presence of Escherichia coli in, 493
Weigert, 6, 114
gentian-violet stain, 88
iodine solution in study of protozoan cysts, 359
Weil, 122
Weismann, 325
Welch, 237
Welch's staining method for capsules, 93
Wells, 87, 122, 123
Wenyon, 345, 346, 347, 360
Wernicke, 7
Wheeler, 121
Wherry, 81
White cell counting, technic of, 457
White diarrhea of chicks, Salmonella pullorum in, 167
Williams, 395
stain for Negri bodies, 93
Wilson, 326
Winslow, 16
Wolff-Eisner, 123
"Wooden tongue," organism of, 245
Woolsorter's disease, organism of, 203
Wright, 108, 109, 464, 465
blood stain, 91
method for determining opsonic index, 109
method of cultivating anaerobes, 75
Wuertz, 265

Yeasts, pathogenic, 301
Yersin, 195

Zenker's fluid, 356
Zettnow's method for staining flagella, 97
Ziehl-Neelsen's carbol-fuchsin stain, 86
method for staining acid-fast bacteria, 90
Zinke, 393
Zinsser, 122
method of cultivating anaerobes, 75
Zoff, 16
Zygote, formation in coccidia, 348, 349
of Babesia, 350

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